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THE UNIVERSITY OF ALBERTA

The Effect of Tannins on the Digestibility of Two Isosynthetic Strains of Birdsfoot Trefoil

by

Johanne Chiquette

(C)

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH
IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE
OF DOCTOR OF PHILOSOPHY
IN
ANIMAL BIOCHEMISTRY

DEPARTMENT OF ANIMAL SCIENCE

EDMONTON, ALBERTA
SPRING 1988

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled The Effect of Tannins on the Digestibility of Two Isosynthetic Strains of Birdsfoot Trefoil (*Lotus corniculatus* L.) in *In Vitro*, *In Sacco* and *In Vivo* Trials submitted by Johanne Chiquette in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Animal Biochemistry.

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Co-Supervisor

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Date ..October 30, 1987..

This thesis is dedicated to

Ivy

for her example of courage and generosity!

ABSTRACT

Low tannin (LT) and high tannin (HT) isosynthetic strains of birdsfoot trefoil (*Lotus corniculatus* L.), grown under greenhouse conditions and containing 1% and 5% condensed tannins respectively, on a dry matter (DM) basis, were tested using *in vitro* and *in sacco* digestibility techniques. After 24-h *in vitro* digestion, using homogenized rumen solids and rumen fluid as inoculum, there was less ($P<0.01$) DM disappearance (45.7% vs. 54.4%) from the HT strain. Also, the development of gas pressure was lower ($P<0.01$) and production of volatile fatty acids (VFA) less ($P<0.05$) with the HT strain than with the LT strain. Average DM loss from four replicate nylon bags was also lower ($P<0.01$) for the HT strain than for the LT strain after 8, 12, and 24 h of incubation in the rumen of steers. In a second experiment, the same two strains were grown under irrigated field conditions and contained 1% and 3% condensed tannins, respectively, on a DM basis. Forage was fed fresh daily to six ruminally cannulated Suffolk wethers. Rumen fluid samples were collected before feeding, 3 h and 6 h after feeding for pH, chlorophyll, supernatant protein nitrogen (N), ammonia nitrogen (NH_3-N) and VFA determinations, and protozoa counts. Results from rumen fluid analyses indicate that sheep receiving the HT strain had a similar ruminal pH, a slightly lower ($P>0.05$) chlorophyll concentration, significantly lower ($P<0.01$) supernatant protein N

and NH_3-N concentrations, and consistently but not significantly lower VFA concentrations and higher protozoa counts than the sheep receiving the LT strain. Apparent crude protein digestibility was 8% less in sheep fed the HT strain. However, apparent DM, organic matter and acid detergent fiber digestibilities were not affected by the level of tannin. Plant protein solubility in rumen fluid and subsequent deamination were higher in sheep fed trefoil containing 1% tannin than in those fed trefoil containing 3% tannin, but there were no effects upon overall forage digestibility.

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TABLE OF CONTENTS

Chapter		Page
I.	Introduction	1
	A. Birdsfoot Trefoil	2
	1. Origin	2
	2. Distribution and importance in North America	2
	3. Plant description	2
	4. Plant physiology	3
	5. Comparative culture requirements	4
	6. Cultivars	5
	7. Utilization	
	a) Pasture	6
	b) Hay	7
	8. Nutritive value and digestibility	8
	9. Breeding programs	9
	10. Conclusion	9
	B. Tannins - Chemical Structure and Properties	10
	1. Definition	10
	2. Classification	10
	3. Occurence of condensed tannins	12
	4. Origin of condensed tannins in plants	12
	5. Chemical bonds	13

Chapter Page

C. Tannins - Biological Properties 16

1. Enzyme inhibition 16

2. Microbial inhibition 16

D. Tannins - Effect Upon Forage Utilization 18

E. Bloat - Plant Factors 20

F. Objectives of the Present Study 22

G. Bibliography 23

II. Effect of Tannins on the Digestibility of Two Isosynthetic Strains of Birdsfoot Trefoil using *in vitro* and *in sacco* techniques 28

A. Introduction 28

B. Materials and Methods 31

C. Results 38

D. Discussion 49

E. Bibliography 53

Chapter | Page

III. Effect of Tannin Content in Two Isosynthetic Strains of Birdsfoot Trefoil on Feed Digestibility and Rumen Fluid Composition in Sheep 58

A. Introduction 58

B. Materials and Methods 61

C. Results 67

D. Discussion 74

E. Bibliography 82

IV. General Conclusions 86

A. Bibliography 88

Appendix 89

LIST OF TABLES

Table		Page
II-1	Extent of *in vitro* dry matter disappearance, gas pressure increase and volatile fatty acid (VFA) production of birdsfoot trefoil strains after 24 h incubation	39
II-2	Extent of *in vitro* dry matter disappearance, gas pressure increase and volatile fatty acid (VFA) production for observations restricted to experimental days where all three variables were measured on birdsfoot trefoil forage containing two different concentrations of tannins	40
II-3	*In sacco* dry matter disappearance (%) of birdsfoot trefoil strains	42
III-1	Mean composition (% of DM) of the two birdsfoot trefoil strains (collected throughout the regrowth)	66
III-2	Digestion of birdsfoot trefoil strains (stems and leaves) upon 24 h incubation in nylon bags suspended in the rumen of a steer	68

Table | Page

III-3 Rumen fluid variables measured before feeding, 3 and 6 h after feeding, in sheep fed high and low tannin birdsfoot trefoil (average over the three feeding periods) .. 69

III-4 Individual volatile fatty acid (VFA) concentrations over sampling times 71

III-5 Apparent digestibilities (%) of birdsfoot trefoil strains in sheep 72

LIST OF FIGURES

Figure		Page
I-1	Gallic acid	11
I-2	1,3,6-trigalloylglucose (hydrolyzable tannin)	11
I-3	Catechin; leucocyanidin	11
I-4	General metabolic pathway	14
I-5	Self condensation of catechin	14
II-1	*In sacco* digestion of high and low tannin strains of birdsfoot trefoil showing the almost complete disappearance of leaf material from the low tannin strain after 24 h	43
II-2	Scanning electron microscopy (SEM) of the surfaces of leaves of the high (A) and low (B) tannin strains of birdsfoot trefoil exposed to digestion for 4 h *in sacco* in the bovine rumen	45

Figure Page

II-3 Transmission electron microscopy (TEM) of ruthenium red-stained preparations of stems of high (A) and low (B) strains of birdsfoot trefoil exposed to digestion for 24 h *in sacco* in the bovine rumen 47

I. INTRODUCTION

Over 200,000 hectares of birdsfoot trefoil are grown in Canada, mainly in the eastern provinces of Ontario and Quebec (Grant and Marten 1985). Birdsfoot trefoil yields are 50 to 80% of those of alfalfa (*Medicago sativa* L.) which is a major reason for its limited hectarage (Grant and Marten 1985). This perennial forage legume has been slowly gaining acceptance in western Canada (Goplen 1969) and may find greatest use where bloat is a problem, since birdsfoot trefoil is a bloat-safe legume, or in those areas where alfalfa cannot be grown because of inadequate drainage or excessive acidity (Goplen personal communication).

Birdsfoot trefoil contains condensed tannins which have been shown to play a role in bloat prevention by complexing with soluble plant proteins, known as foam stabilizers, in the rumen (Mangan 1959). Another beneficial effect of tannins, due to their protein-binding property, resides in the reduction of ruminal plant protein degradation and the increase in amino acid supply to the animal (Barry and Reid 1984). However, many studies have demonstrated the detrimental effects of high concentrations of tannins on forage dry matter (DM) digestibility *in vitro* (Cope and Burns 1971; Marquardt et al. 1978), *in sacco* (Donnelly and Anthony 1969, 1970) and on readily fermentable carbohydrates and hemicellulose digestibilities *in vivo* (Barry and Manley 1984).

This introduction will review the agronomic characteristics of birdsfoot trefoil legume as well as the chemical and biological properties of tannins and their effect upon forage utilization.

A. BIRDSFOOT TREFOIL

1. Origin

Species of Lotus are widely distributed throughout the world. The greatest diversity occurs in the Mediterranean basin, indicating that this region was probably the center of origin (Seany 1973).

2. Distribution and importance in North America

In the United States, birdsfoot trefoil is adapted to regions ranging from the Atlantic Coast to the North-Central United States (Kansas, Nebraska, Dakotas) and south to North Carolina, Tennessee, and northern Arkansas (Seany and Henson 1970). The hectarage has steadily increased from about 260,000 ha in 1957 to the present estimate of close to one million ha (Grant and Marten 1985). Because of wide adaptation, persistence and relatively high forage yield, birdsfoot trefoil has replaced much of the red clover (*Trifolium pratense* L.) and white clover (*Trifolium repens* L.) previously grown in the Northeast. In the Midwest, it has gained importance as a persistent and productive permanent pasture legume when grazed by cattle or sheep. Over 200,000 ha are grown in Canada, mainly in the eastern provinces of Ontario and Quebec (Grant and Marten 1985).

3. Plant description

Birdsfoot trefoil is a perennial forage legume. It has finer, softer, and leafier stems than alfalfa and a lower cellulose content but more of the other carbohydrates (Duke et al. 1981). Its long taproot with numerous lateral branches does not penetrate

as deeply as that of alfalfa but the distribution of branch roots in the upper soil is more extensive (Seany 1973).

Although flowers are perfect, having stamens and pistils, most seed results from cross-pollination (Grant and Marten 1985). An average of five to six pods are attached at right angles to the end of the flower stem, giving the appearance of a bird's foot, thus, the common name birdsfoot trefoil (Seany and Henson 1970). Seeds are very small, 825,000-925,000/kg (Grant and Marten 1985).

Birdsfoot trefoil is a legume that does not cause bloat in cattle. This bloat-safe characteristic has been attributed to the presence of condensed tannins and to the high resistance of birdsfoot trefoil's cells to rupture (Lees et al. 1981; Howarth et al. 1982).

4. Plant physiology

Smith (1962) observed that carbohydrate reserves in trefoil roots were reduced during the spring and remained at a low level during summer. Accumulation occurred only when vegetative growth ceased in autumn. In contrast, root reserves in alfalfa were reduced during the spring and after each cutting and were restored as the top growth approached maturity. Thus, birdsfoot trefoil regrowth after cutting is primarily dependent on carbohydrates synthesized by top growth left after cutting, rather than on reserves stored in the roots as with alfalfa.

Legumes fix nitrogen from the air through symbiotic rhizobial bacteria living in nodules on their roots. These rhizobia get their food from the legume and in return, provide it with nitrogen (Cooper et al. 1971). Inoculation is necessary for successful stands in areas where trefoils have not been grown before.

Bacteria used to inoculate alfalfa, red clover, and other common legumes are not effective on trefoils (Duke et al. 1981).

Birdsfoot trefoil's acceptance has been impeded because of its lack of seedling vigor (Cooper 1966). Its seedlings exhibit slow emergence in the spring, as well as poor competition with other plants since they are slow to establish (Twamley 1969). Seedling vigor has been positively associated with seed size or weight (Grant and Marten 1985). It is also slow to recover following clipping and grazing (Cooper et al. 1971). The latter authors have reported birdsfoot trefoil to be less winterhardy than alfalfa but more than ladino clover (*Trifolium repens* L.).

5. Comparative culture requirements

Birdsfoot trefoil has been reported to grow better on poorly drained clay soils than alfalfa but to be less drought tolerant, which is explained by its shorter taproot as well as the extensive distribution of its branch roots in the upper soil (Cooper et al. 1971; Seany and Henson 1970). Its adaptation to poorly drained soils has been of great importance in the Northeast and in the North Central United States which have large hectarages too wet for growing alfalfa (Seany 1973). It is also more drought resistant and winterhardy than ladino clover and requires less exacting soil fertility and moisture conditions than most legumes (Cooper et al. 1971). However, McDonald (1946), who studied the effect of various fertilizers and lime on the growth of birdsfoot trefoil, concluded that while it tends to produce better than other legumes on normally poor soils, the soil-fertility requirements of

trefoil did not differ from those of commonly used legumes. Winch (1958) reported that birdsfoot trefoil has been successfully grown on soils which have a pH of 5.0 to 6.0, the optimum pH for maximum growth being 6.0 as compared with 6.5 to 6.8 for alfalfa and red clover.

6. Cultivars

Two distinct types of birdsfoot trefoil are grown in the U.S. and in Canada, the New-York or Empire type discovered in Albany County, NY, and the European type introduced from Europe (Seany 1973). The primary difference between these types is their growth habit, maturity, seedling and recovery growth rates, and winter-hardiness (Seany and Henson 1970).

The Empire cultivar was developed at the Cornell University Agricultural Experiment Station (Seany and Henson 1970). It is characterized by a very fine and weak stem, lateness in maturity, and a prostrate habit of growth. It is as winterhardy as alfalfa (Winch 1958) and, under severe winter conditions, tests in Minnesota and Canada have shown Empire to be more winterhardy than European type cultivars (Grant and Marten 1985). However, this cultivar lacks seedling vigor, its recovery after harvest is slow and its yield is low (Grant and Marten 1985).

On the other hand, the European type cultivars have an erect type of growth, mature 10-14 days earlier, possess greater seedling vigor, earlier spring growth, and a more rapid recovery after cutting than Empire, but are not as winterhardy (Grant and Marten 1985).

Many cultivars of this European type have since been developed. Sufficient winterhardiness has been incorporated to meet the needs

of the specific areas where they were bred (Winch 1958). At least 25 cultivars have been developed in the U.S. and Canada from germplasm within the wild species (Grant and Marten 1985).

The Cornell University Agricultural Experiment Station developed the cultivar Viking, a European type, erect, early maturing, and superior in winterhardiness to imported lots of trefoil (Seany and Henson 1970).

The cultivar Leo was developed at MacDonald College in Quebec and is shown to have excellent early spring vigor and greater winterhardiness than either Empire or Viking (Seany and Henson 1970).

Cree is another winterhardy strain of birdsfoot trefoil selected at Saskatoon. Because of its high forage and seed yields, and hardiness, Cree is recommended along with Leo in western Canada, in preference to the older standard varieties of Empire and Viking (Goplen personal communication).

7. Utilization

a) As pasture

Many studies have shown birdsfoot trefoil to be an excellent pasture legume. It has a major advantage over most other legumes in that it does not cause bloat. In general, renovating and seeding birdsfoot trefoil in unimproved permanent pastures has greatly improved animal gains (Seany and Henson 1970). Marten and Jordan (1979) reported higher lamb gains when birdsfoot trefoil was included as one of three paddocks in a rotational grazing system of alfalfa-grass mixtures. Marten and Ehle (1984) reported

higher heifer daily gains with birdsfoot trefoil than with alfalfa and concluded from a 3-year grazing trial that birdsfoot trefoil pasture was superior to alfalfa, sainfoin (*Onobrychis viciifolia* Scop.), and cicer milkvetch (*Astragalus cicer* L.) pastures in their environment. Smith and Nelson (1967) reported that under natural conditions, Empire trefoil is a legume that can persist better than alfalfa when heavily and frequently grazed or cut. The shoots of alfalfa grow almost upright, while those of Empire trefoil are basally prostrate. Thus, close and frequent cutting removes nearly all of the leaf area of alfalfa, but many leaves will remain on the prostrate stubble of trefoil to assure vigorous regrowth after grazing (Grant and Marten 1985).

Because birdsfoot trefoil blooms intermittently throughout the summer, it often sets seed on low-growing stems, even when closely grazed. Thus, reseeding also contributes to its persistence (Hoveland et al. 1982).

Mays and Washko (1960) reported that birdsfoot trefoil was particularly good for stockpiling, the practice by which forage is allowed to accumulate in the field through the summer and early fall for late fall grazing, because it retains its high quality leaves on mature growth better than does alfalfa.

In addition to being used as a major pasture legume, it is used extensively on new highway slopes for improving the soil and controlling erosion (Duke et al. 1981).

b) As hay

Birdsfoot trefoil carbohydrate root reserves decrease during spring growth and remain relatively low until the end of the

growing season. Thus, regrowth after hay harvest is dependent on photosynthesis. Therefore, birdsfoot trefoil cannot be completely defoliated as frequently as alfalfa or stands will quickly deplete. This is why birdsfoot trefoil is considered to be better adapted for pasture than for mechanically harvested forage production (Grant and Marten 1985).

Birdsfoot trefoil is not a highly productive hay crop (Cooper et al. 1971). Yields usually are 50-80% that of alfalfa (Grant and Marten 1985). At Bozeman, Montana, Cooper et al. (1971) reported that it produced less than alfalfa, as much as red clover and more than ladino clover in a 4-year period. Since it is very fine-stemmed, it also lodges easily (Cooper et al. 1971). However, the quality of birdsfoot trefoil hay is excellent and compares favorably with that of alfalfa and other good legume hay (Cooper et al. 1971; Seany and Henson 1970). The more erect growing varieties of European origin are more productive and better suited for hay than the Empire cultivar (Seany and Henson 1970; Duke et al. 1981).

8. Nutritive value and digestibility

In composition and nutritive value, birdsfoot trefoil compares favorably with alfalfa and white clover. Duke et al. (1981) reported that the green forage (23.3% DM) contained on a moisture-free basis: 14.8-29.1% crude protein (avg. 21.6), 1.2-5.0% fat (avg. 2.5), 13.0-28.4% crude fiber (avg. 22.2), 5.6-10.5% ash (avg. 8.1) and 43.8-49.9% N-free extract (avg. 45.6).

Dry matter intake and digestibility as well as crude protein digestibility of birdsfoot trefoil hay have equaled those of alfalfa and other good legume hays when fed to sheep (Ingalls et al. 1965) and to dairy cows (Loosli et al. 1950). However, Loosli et al. (1950) found that crude fiber digestibility of birdsfoot was lower than that of alfalfa. These same authors also reported that milk from cows fed trefoil hay contained more carotene, vitamin A and E, and exhibited better keeping qualities than milk from cows receiving ladino clover and timothy (*Phleum pratense* L.) hays. Birdsfoot trefoil resulted in milk productions similar to those of animals fed alfalfa or ladino clover. More recently, Duke et al. (1981) reported that milk from cows fed on trefoil hay contained more vitamins A and E than that from cows given alfalfa hay.

9. Breeding programs

The primary objective of breeding programs in North America has been to increase forage yields of the crop when used for pasture or hay. Specific breeding objectives to accomplish this have involved improvement of seedling vigor, resistance to root rots, winterhardiness and rapid recovery growth after cutting (Seany and Henson 1970).

10. Conclusion

Because birdsfoot trefoil does not cause bloat, this species should be used, particularly for pasture purposes. It will probably find greatest use where bloat is a problem and in those areas where alfalfa cannot be grown because of inadequate drainage or excessive acidity (Goplen personal communication).

B. TANNINS - CHEMICAL STRUCTURE AND PROPERTIES

1. Definition

The term tannin referred originally to substances responsible for the conversion of raw animal skins into leather (Haslam 1966). It is now generally used to include any naturally occurring compound of high enough molecular weight (500-3000) and containing a large number of phenolic hydroxylic groups to enable it to form effective crosslinks with proteins and other macromolecules (Swain 1965). In the tanning process, collagen fibers of the corium of skin are cross-linked by the tannins in plant extract causing the skin to harden and shrink to give leather (Haslam 1966).

2. Classification

Tannins are classified into two groups: the hydrolyzable and the condensed tannins which correspond to two well differentiated structural types. Hydrolyzable tannins consist of a carbohydrate core (usually glucose, but in some cases polysaccharides have been identified) whose hydroxyl groups are esterified by phenolic acids (e.g., gallic acid) or their derivatives (Figs. I-1 and I-2). These polyesters are readily hydrolyzed either by acids, bases, or enzymes. Condensed tannins, also called flavolans, are formed by the condensation of two or more molecules of flavan-3-ols, such as catechin (Fig. I-3A), or flavan-3,4-diols, such as leucocyanidin (Fig. I-3B), or mixtures of the two. Condensed tannins do not readily break down by acid treatment but instead polymerize to yield insoluble, amorphous, often red-colored products known as phlobaphenes (Swain 1965).

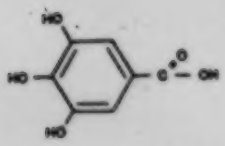

Figure I-1. Gallic acid (Ribereau-Gayon 1972).

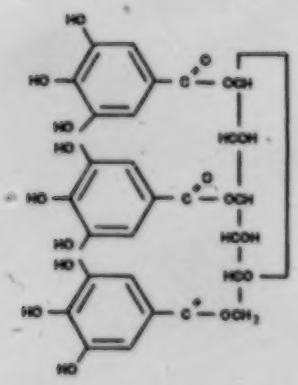

Figure I-2. 1,3,6-trigalloylglucose (hydrolyzable tannin) (Kraiem 1985).

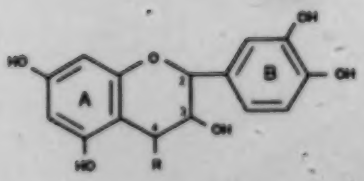

Figure I-3. (A) R=H; catechin (Ribereau-Gayon 1972)

(B) R=OH; leucocyanidin.

3. Occurrence of condensed tannins

Of the two classes of tannins, the condensed tannins are the more widely distributed in higher plants (Schanderl 1970). Condensed tannins occur in the leaves and stems of sainfoin and of Lotus species, in the flower petals only of white clover and red clover, and are not detectable in alfalfa and in grasses (Jones et al. 1976). Swain (1979) reported that some herbaceous legumes (e.g., *Lathyrus pratensis*) may contain up to 16% extractable tannins in their leaves, but more usually, truly tanniferous plants accumulate 5-10% of the polymers in various organs. Tannin concentrations as high as 8-10% of DM have been reported by Barry and Forss (1983) in *Lotus pedunculatus* Cav., when grown in low fertility acid soils under cold conditions. Similar concentrations (12% of DM) have also been found in immature leaves of sericea lespedeza (*Lespedeza cuneata* [Dumont] G. Don) (Burns 1966). These concentrations are among the highest reported in legume forages grown under field conditions. Cultivars of birdsfoot trefoil contain lower concentrations of condensed tannins (0.2-3% of DM) (Ross and Jones 1983), while John and Lancashire (1981) reported condensed tannins in sainfoin to be 6% of DM. In birdsfoot trefoil, condensed tannins are present in organelles separated from the cytoplasm. The tannin vesicles are located just under the epidermal layer, frequently adjacent to the stomata (Lees et al. 1982).

4. Origin of condensed tannins in plants

Isotopic tracer studies (Srinavasan et al. 1959; Haslam 1966) have demonstrated that the production of the flavanoids agrees with

the general hypothesis in which C_6 unit (ring A) is acetate derived and that the C_6-C_3 unit (ring B) has its origin in the shikimic acid pathway (Figs. I-3 and I-4). A number of alternative routes for the condensation-polymerization reactions of flavan-3-ols (Fig. I-5) and flavan-3,4 diols have been considered varying from the acid catalyzed mechanism (Haslam 1966) to the oxidative polymerization discussed by Hathway and Seakins (1957). Although flavanoids occur abundantly in some plants and are present to some extent in all plant tissues, hardly anything is known of their breakdown or turnover by plant enzymes (Swain 1965).

5. Chemical bonds

The essential property of tannins is their ability to combine with proteins and other polymers such as cellulose and pectin (Swain 1965). Swain (1965) and Loomis and Battaile (1966) suggested that the formation of the tannin-protein or other polymer combinations involves three types of bonding: A) Hydrogen bonds between the phenolic group of the tannins and receptor groups (-NH-, -CO-, or -OH) of the protein or other polymers. Tannins have been found to have a greater affinity for protein than for cellulose, which has been attributed to the strong hydrogen bond affinity of the carbonyl oxygen of the peptide group (e.g., $^{+}NH_3$-CH_2-CO-NH-CH_2-COO^{-}) (Russel et al. 1968b). This condensed tannin-protein complex is stable and insoluble in the pH range of 3.5-7.0 but is unstable and releases protein at pH <3.0 and at pH 8.0 (Jones and Mangan 1977). B) Ionic bonds between anionic groups of the tannins (ionized phenolic or carboxylic groups) and cationic groups of the proteins

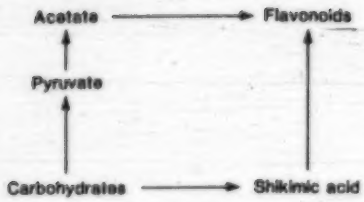

Figure I-4. General metabolic pathway (Robinson 1963).

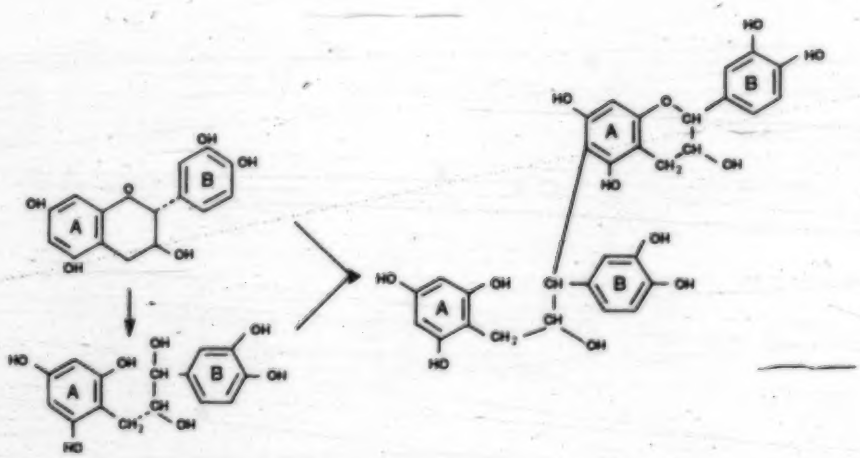

Figure I-5. Self condensation of catechin. (Haslam 1966; Ribereau-Gayon 1972).

influence tannin binding of substrates particularly with the hydrolyzable tannins which have numerous carboxyl groups (Russel et al. 1968a). C) Covalent links may be formed when the protein and polyphenol are brought into contact under some conditions (e.g., alkaline pH, O_2) then the polyphenol may become oxidized to a quinone and form covalent linkages with protein (Kumar and Singh 1984). These latter bonds impart great stability to the tannin-protein complex (Ribereau-Gayon 1972). D) A fourth type of bonding was suggested by Loomis (1974) and Hagerman and Butler (1981) which consists of a hydrophobic interaction between the aromatic ring structure of phenolic compounds and hydrophobic regions of protein.

C. TANNINS - BIOLOGICAL PROPERTIES

1. Enzyme inhibition

Tannins are a potent inhibitor of digestive enzymes due to their capacity to bind with enzyme proteins as well as with the substrate (Benoit and Starkey 1968). The latter observed a marked inhibitory effect of wattle tannin on *in vitro* decomposition of rye cellulose and hemicellulose by soil microorganisms. They reported that although it seemed likely that the principal effect of tannin on cellulose decomposition was interference with cellulase activity, the tannin might have had an additional effect as a result of its direct reaction with cellulose, since it was found to be absorbed by cellulose which became brown in tannin solution. Porter and Schwartz (1962) isolated a water-soluble substance from grape leaves which had been shown to inhibit cellulase and found it to be a tannin.

Studies dealing with the effect of tannins on ruminal enzymatic reactions agreed with *in vitro* observations. Hence, Smart et al. (1961) observed that enzymatic hydrolysis of cellulose was inhibited by part of the tannin fraction present in the leaves of Sericea lespedeza while Kumar and Singh (1984) reported the inhibition of rumen fluid urease activity by aqueous extracts of oak (*Quercus incana*) leaves.

2. Microbial inhibition

Most organisms are sensitive in some degree to the inhibitory effects of tannins. However, tannins of different origin and composition will affect microorganisms differently (Henis et al., 1964).

White (1957) reported that tannin substance seems to protect vulnerable parts of the plant from microbial attack by inactivating viruses and invasive extracellular enzymes via a direct tanning action. In that respect Cadman (1960) indicated that tannins prevented the infection of plants by tobacco mosaic virus by adsorbing on the virus surface. Henis et al. (1964) suggested that one of the early effects exerted by tannins on sensitive bacteria was the inhibition of cell-wall synthesis, as demonstrated by the formation of "protoplasts" (McQuillen 1960) by *Cellvibrio fulvus*, a cellulolytic bacteria, in the presence of tannic acid or a tannin fraction of carob extract.

D. TANNINS - EFFECT UPON FORAGE UTILIZATION

Because of the ability of tannin to complex with proteins as substrates or enzymes, it is to be expected that the digestion by the ruminant of the fibre and protein of a forage containing tannin would be depressed (McLeod 1974).

Arora et al. (1975) found *in vitro* DM disappearance to be negatively correlated with tannin content of sorghum (*Sorghum bicolor* L.) forage, which observation was also verified in crownvetch (*Coronilla varia* L.) forage by Burns and Cope (1974). Cope and Burns (1971) reported that *in vitro* DM disappearance of a high tannin (HT) strain of *Lespedeza cuneata* was consistently about 25% lower than that of the low tannin (LT) strain, over years, harvests, and locations. Donnelly and Anthony (1970), also working with two strains of *L. cuneata*, but using the nylon bag or *in sacco* technique, showed the adverse effect of tannin on disappearance of DM (58.5% and 64.5%) from the HT and LT strain, respectively.

In a feeding trial study using *Lotus pedunculatus* Cav. containing 4.6% and 10.6% DM of total condensed tannins fed fresh to sheep at hourly intervals, Barry and Manley (1984) found that apparent digestibility of hemicellulose, soluble carbohydrate and pectin was less for HT than for LT Lotus. In a recent study, Barry et al. (1986) sprayed *L. pedunculatus* with polyethylene glycol (PEG) (which selectively binds to tannins and prevents them from binding proteins) at three concentration levels, in order to get dietary concentrations of condensed tannins of 1.4%, 4.5% and 9.5% (DM). They reported that increasing tannin concentration linearly decreased the apparent digestibility of organic matter and rumen digestion of hemicellulose.

In view of the fact that stability of the condensed tannin-protein complex is strongly pH dependent (Jones and Mangan 1977), being insoluble and stable in the pH range 3.5-7.0 but dissociating and releasing soluble protein at pH <3.0 and at pH 8.0, the presence of condensed tannins in fresh forages should, therefore, theoretically reduce plant protein degradation rates in the rumen (pH 5.8-6.8), yet allow their solubilization and release in the abomasum (pH 2.5-3.5) and small intestine (pH 7.5-8.5). John and Lancashire (1981) confirmed this as they reported a 15% decrease in microbial nitrogen (N) and a 36% increase in plant N leaving the stomach of sheep fed on *L. corniculatus* cv. Maitland, which contained 1.45% (DM) condensed tannins, compared with cv. Empire (0.25% condensed tannins). These results were further supported by Barry et al. (1986), who reported that increasing dietary condensed tannin concentration linearly increased duodenal flow of non-ammonia nitrogen. However, none of these studies determined the amino acid fluxes through the abomasum.

It was only recently that Waghorn et al. (1987), comparing *L. corniculatus* containing 2.2% condensed tannins (DM) with Lotus given to sheep receiving intraruminal PEG infusion to bind tannins, reported that: 1) Rumen ammonia concentrations were lower in control sheep than in PEG sheep. 2) The flux of essential amino acids through the abomasum of control sheep was 50% greater than that in PEG sheep, while the flux of non-essential amino acids (NEAA) was 14% higher in control than in PEG sheep. 3) The presence of tannins in the control group increased net apparent absorption of threonine, valine, isoleucine, leucine, tyrosine, phenylalanine, histidine, and lysine and reduced NEAA absorption by 10%, compared with PEG sheep.

E. BLOAT

1. Plant factors

The soluble proteins present in rumen contents of cattle grazing lush pasture such as white clover, red clover or alfalfa have been implicated as surfactants (surface active agents) of major importance responsible for the stable foams that develop in the rumen of animals suffering from bloat (Mangan 1959). Tannins play an important role in bloat prevention by complexing these soluble proteins during disintegration of plant material, such as chewing by animals.

The literature does not report any occurrence of important bloat condition from feeding tannin-containing forages. On the other hand, not all bloat-safe forages contain tannins, since cicer milkvetch and arrowleaf clover (*Trifolium vesiculum* Savi) are tannin-free and do not promote bloat (Howarth et al. 1984). This led researchers to investigate plant factors, other than tannins, involved in bloat prevention. Hence, Fay et al. (1980), using chewed herbage from rumen-fistulated cattle, showed *in vitro* digestion of bloat-causing legumes (alfalfa, white clover and red clover) to be faster and to produce more foam in the digestion flasks than bloat-safe legumes (birdsfoot trefoil, sainfoin and cicer milkvetch). A greater insight as to why differences in bloat-causing and bloat-safe legumes occur was provided by Lees et al. (1981), who subjected whole leaves and isolated mesophyll cells to sonication, homogenization with a ground glass tissue grinder and shaking with glass beads, and reported that birdsfoot trefoil and other bloat-safe legumes: sainfoin and cicer milkvetch, had stronger cell walls either alone or combined with a higher degree of tissue strength than bloat-causing

legumes: alfalfa, white clover and red clover. Howarth et al. (1982), using a modified nylon bag technique, confirmed that leaf tissue disruption by microbes was less rapid in bloat-safe legumes than in bloat-causing legumes.

F. OBJECTIVES OF THE PRESENT STUDY

A review of the literature showed that Grasslands Maku (*Lotus pedunculatus* Cav.), a Lotus cultivar adapted to New Zealand's conditions, was the main forage used in feeding trials dealing with the effect of tannins on forage digestibility. Furthermore, in most studies LT and HT Lotus were obtained by either spraying forage with PEG (Barry et al. 1986) or infusing it intraruminally (Waghorn et al. 1987). The effects attributable to tannins were also assessed by growing plants under high and low levels of soil fertility, inducing low and high concentrations of tannins respectively (Barry and Duncan 1984; Barry 1985). These two methods, however, present some serious disadvantages in that PEG treatment increases the apparent concentration of lignin and increases the concentration of water-soluble carbohydrates in the plant and reduces its rate of degradation in the rumen (Barry et al. 1986), while fertilizer application increases plant total nitrogen content and increases plant DM yield.

In the present study, two isosynthetic strains of LT and HT birdsfoot trefoil were tested for the first time. The objectives of this study were to determine the effects of tannins on *in vitro* and *in sacco* digestion of birdsfoot trefoil. Both strains were also compared when fed to sheep regarding their effect on rumen fluid variables as well as organic matter (OM), crude protein (CP), and acid detergent fiber (ADF) apparent digestibilities.

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II. EFFECT OF TANNINS ON THE DIGESTIBILITY OF TWO ISOSYNTHETIC STRAINS OF BIRDSFOOT TREFOIL USING *IN VITRO* AND *IN SACCO* TECHNIQUES

A. INTRODUCTION

Birdsfoot trefoil is extensively grown for pasture and hay in the North-Central and North-Eastern United States and Eastern Canada (Seaney and Henson 1970). This perennial forage legume is tolerant of waterlogged, saline, acidic, and calcareous soil conditions (Laskey and Wakefield 1978). Its feeding value was reported to equal that of alfalfa and other good legume hays when fed to sheep (Ingalls et al. 1965) and dairy cows (Loosli et al. 1950). Marten and Jordan (1979) reported an increased feeding value when one-third of an alfalfa-grass pasture was substituted with birdsfoot trefoil.

Birdsfoot trefoil forage contains secondary plant metabolites called "condensed tannins" that confer both beneficial and detrimental effects on forages. Condensed tannins are polymeric flavanols (gallocatechins and catechins) that react most strongly by hydrogen bonding with proteins (Barry and Reid 1984), and result in a reduction of plant protein degradation by rumen microbes. This can enhance post-ruminal absorption of plant amino acids by the animal (Reid et al. 1974; Ulyatt et al. 1977).

Several studies (Jones et al. 1970; Gutek et al. 1974; Swain 1979) have confirmed the formation of reversible complexes of tannins with soluble proteins from trefoil leaves. Plant soluble proteins released in the rumen are known as foam stabilizers, which foam is responsible for trapping gas bubbles and hence causing bloat (McLeod 1974).

Therefore, tannins, by complexing soluble proteins may be involved in bloat prevention. However, cicer milkvetch and arrowleaf clover contain no tannins and are known for not causing bloat (Howarth et al. 1984). Obviously other factors operate in the etiology of pasture bloat. Howarth and coworkers (1979) found strong evidence that the mechanical strength of leaf tissue and its resistance to microbial digestion could also be responsible for preventing bloat. Lees et al. (1981) confirmed that birdsfoot trefoil and other bloat-safe legumes: sainfoin and cicer milkvetch, had either stronger cell walls or a higher degree of tissue strength than bloat-causing legumes: alfalfa, white clover and red clover.

Howarth and coworkers (1979, 1982), using nylon bag digestion techniques for bloat-causing and bloat-safe forage legumes, showed an increased rate of DM disappearance from bloat-causing legumes: alfalfa, red clover, and white clover, as compared with bloat-safe legumes: birdsfoot trefoil, sainfoin and cicer milkvetch. Fay et al. (1980, 1981) reported that bloat-causing forages are more rapidly and more heavily colonized by rumen bacteria. Marquardt's investigation (Marquardt et al. 1978) on two cultivars of fababeans (tannin-free and tannin-containing) indicates a negative correlation between the *in vitro* DM digestibility and the content of condensed tannins. These studies dealt with comparisons of legumes showing variations in other constituents in addition to tannins. In an earlier study on the effect of tannins on forage quality, Cope and Burns (1971) used two synthetic strains of sericea, differing in tannin levels but quite similar in fiber and lignin concentration, and reported that the DM disappearance of the LT strain was 25% greater than that of the HT

strain. However, the tannins present in sericea are different from those of birdsfoot trefoil in that they are of larger molecular weight, unpalatable and antinutritive. Furthermore, their production is controlled by many gene pairs (Bates and Henson 1955). Results from Dalrymple's work (1982) show a high heritability for the tannin character in birdsfoot trefoil, the F_1 plants from crosses between HT and LT parents were all high in tannin, thus indicating dominance for tannin production. Moreover, F_2 and backcross populations demonstrated that the tannin character was controlled by a single dominant gene. Of 511 F_2 plants, 383 exhibited HT content, while 128 showed LT content, providing a good fit to a 3:1 ratio for single gene inheritance. These results confirm suggestions reported in a D.S.I.R. report in 1980 which indicate a single gene difference between tannin-positive and tannin-negative types of Lotus. Therefore, it was of interest to study the effect of the specific tannins present in birdsfoot trefoil on the nutritive value of this forage by using the *in vitro* and *in sacco* digestion techniques.

B. MATERIALS AND METHODS

Plant Material

The present study was conducted on two isosynthetic strains of birdsfoot trefoil. The term "isosynthetic" as used in this work refers to similar genetic populations differing in one contrasting character. Although this concept was independently conceived by Goplen (1969), it coincides with Burton's (1966) "near-isogenic populations". However, the term isosynthetic is more specific and descriptive and can be applied equally well to both cross-pollinated and self- pollinated crops. In addition, it avoids confusion with the closely allied terms "isogenic" and "near-isogenic" lines which are commonly applied to self-pollinated crops.

The simplicity and speed of developing near-isogenic populations (isosynthetics) has been clearly outlined by Burton (1966). Birdsfoot trefoil isosynthetic strains were developed at the Agriculture Canada Research Station in Saskatoon, following Dalrymple and coworker's study (1984) on the genetic basis for the control of tannin production in birdsfoot trefoil, where it was found that tannin content was controlled by a single dominant gene. The breeding methods used to develop these two strains (Goplen 1969), as well as the type of selection based solely on tannin content among an F_2 population, make these two strains very similar genotypically, thus, their identification as isosynthetic strains. The two parental cultivars were "Cascade" as the HT cultivar and "Cree" as the LT cultivar. The selection was based on several consecutive qualitative Vanillin-HCl spot tests, where leaves were crushed between two layers of Whatman 0.16 mm chromatography paper, following the method of Sarkar and

Howarth (1976). A positive reaction was indicated by the presence of varying shades of pink to magenta. Tannin content of plants was scored 0 to 5 as follows: 0 = no tannins, 1 = trace, 2 = low, 3 = medium, 4 = high, and 5 = very high. Plants selected as HT scored 5 while LT scored 0 to 1.

Birdsfoot trefoil seeds from these isosynthetic strains were grown in the greenhouse for use in the present study. The two strains were similar phenotypically. Quantitative tannin content determinations were performed on each strain for further selection. Preliminary analyses on ten LT and HT birdsfoot trefoil plants, from which stems and leaves were analyzed separately, showed that stems contained four to five times less tannins than leaves. Since a very small sample (200 mg) is required, only leaves (where most of the tannins are) were used for quantitative tannin analysis.

For each plant used in the *in vitro* and *in sacco* experiments, 1 to 2 grams of fresh leaves were stripped off the stems and stored as freeze-dried solids under desiccants in the dark at 5°C, under which conditions tannins are stable (Broadhurst and Jones 1978). Freeze-dried leaves were ground through a 1-mm mesh screen and quantitative tannin analysis was performed using the modified vannilin-HCl method (Dalrymple 1982). Over one-hundred plants were analyzed for tannins in this study and no important interference by anthocyanins and dihydrochalcones was detected, though their presence has been reported in birdsfoot trefoil by Sarkar and Howarth (1976). The interference was examined by preparation of a sample blank containing the tannin extract and a control solution (1% HCl in methanol) lacking the reactive agent (vanillin). There was a wide

range in tannin content within each strain, however, plants showing extremes of tannin concentration were selected for both *in vitro* and *in sacco* experiments, in order to avoid possible overlapping in tannin content between strains.

Only two-year old birdsfoot trefoil plants were used in the *in sacco* studies. Altogether, twenty plants (ten of each strain) were equally divided in two batches of plants. Each batch contained five plants of LT and HT birdsfoot trefoil and was cut five times at four- to five-week intervals. New seedlings as well as two-year old birdsfoot trefoil plants were used in the *in vitro* experiments. New seedlings consisted of fifty plants (twenty-five of each strain) equally divided in five batches of plants. Each batch contained five plants of LT and HT birdsfoot trefoil and was cut twice at five-week intervals. One of the two batches of two-year old birdsfoot trefoil described earlier was cut three times at five-week intervals for the *in vitro* experiments.

All these plant groups were formed to provide plants with appropriate maturity for cutting on successive experiments conducted twice weekly. The upper 10-15 cm of the plants were cut in the bud to 10% flower stages of growth and contained on average 20% DM. The plants were chopped to approximately 2.5 cm long. Buds and flowers (when present) were discarded so that only stems and leaves were used for all experiments.

In Vitro Procedure

Microbial Inocula. Rumen fluid and solid contents (Fay et al. 1980) were collected before morning feeding from a fistulated Holstein steer

fed cubed alfalfa hay. Three hundred grams of solid contents were combined with 300 mL of rumen fluid in a Waring Blender and mixed at half speed for 30-sec and at full speed for another 30-sec period. The homogenate was then squeezed through two layers of cheesecloth into a 4-L Erlenmeyer flask and stirred. For each *in vitro* experiment and each birdsfoot trefoil strain, five plants were chopped, well mixed and 10 g (fresh weight) was introduced into each of six round bottom digestion flasks to which 300 mL of inoculum was added after removal of foam. Carbon dioxide gas free of O_2 was flushed onto the inoculum throughout its preparation to maintain anaerobiosis. The anaerobic technique used throughout the experiment was essentially that of Hungate (1950) as modified by Bryant and Burkey (1953). After inoculation of the plant material, the digestion flasks were stoppered and put in a shaking water bath at 38-39°C. The incubation was carried out anaerobically (under an initial 100% CO_2 atmosphere) for 24 h. A sample of fresh plant material was oven-dried (70°C overnight) for DM determination.

Gas Production. Measurements of pressure, within digestion flasks, as an indication of gas production were taken at the conclusion of the incubation period. A needle connected to a tensiometer was inserted through the rubber stopper of the digestion flask and the pressure was read in millibars (1 millibar = 0.1 kPa).

Volatile Fatty Acid (VFA) Determination. Samples (2 mL) from the digestion flasks and from a blank consisting of inocula without plant material were deproteinised overnight with 24% (wt/vol) m-phosphoric

acid in 5N sulfuric acid (0.4 mL) and centrifuged at 7700 g for 30 min. The supernatant was mixed with an equal volume of crotonic acid (30 mM) as an internal standard. A 1-uL subsample was injected onto a column of the type 6% free fatty acid phase (FFAP, Porapak Q 80/100) on a Varian model 3700 gas chromatograph, equipped with a Shimadzu chromatopac C-RIB data processor.

DM Disappearance. Residual plant material recovered after 24 h incubation with rumen inoculum was poured onto a sieve, washed with tap water until the water was clear and oven-dried at 70°C overnight. DM disappearance was calculated as the difference in the forage DM before and after incubation. Altogether, thirteen *in vitro* experiments were conducted, in five of these there was complete measurement of gas release, VFA concentration and DM disappearance.

***In Sacco* Procedure**

Two fistulated Holstein steers fed cubed alfalfa hay were used. The plant material was prepared as in the *in vitro* procedure. Thirty-two 15-g fresh samples, sixteen from each of the two strains of birdsfoot trefoil, were placed in nylon bags (Nitex No. 130, 10-N, 0.13 mm opening; B&SH Thompson & Co. Ltd., Scarborough, Ontario). Four bags of the LT and four of the HT strain were attached, alternating low and high samples, to each of four weighted bottles designed to keep samples immersed in rumen fluid. Two bottles with their attached bags were placed in the ventral part of the rumen of each of the two fistulated steers. At 4, 8, 12, and 24 h, one bag of each strain was removed from each of the bottles in both steers. Tap water was

immediately run through each bag until the water was clear, after which the bags and contents were oven-dried at 70°C overnight. DM disappearance was calculated as in the *in vitro* experiment. Altogether ten *in sacco* experiments were conducted.

Sample Preparation for Scanning Electron Microscopy (SEM). Samples of the two strains of birdsfoot trefoil, representing each of the four incubation times, were prepared for SEM fixation by soaking for 1 h in 0.5% (vol/vol) glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) and for 2 h in 5% (vol/vol) glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2). After five washes with cacodylate buffer, the samples were treated with thiocarbohydrazide and OsO_4 by the method of Mallick and Wilson (1975) to induce conductivity, dehydrated in ethanol, dried by the critical point method (Cohen et al. 1968), mounted on aluminum stubs, and examined with a Hitachi-450 scanning electron microscope.

Sample Preparation for Transmission Electron Microscopy (TEM)
Samples of the two strains of birdsfoot trefoil, representing each of the four incubation times, were stained for TEM by soaking for 1 h in a solution containing 0.15% (wt/vol) ruthenium red and 0.5% (vol/vol) glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2), and for 2 h in 0.05% (wt/vol) ruthenium red and 5% (vol/vol) glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2). After five washes with cacodylate buffer, the samples were post-fixed in 2% OsO_4 in cacodylate buffer with 0.05% (wt/vol) ruthenium red and washed five times in this buffer before dehydration in ethanol and embedding in Spurr Low Viscosity Embedding medium. Sections were cut, stained with uranyl acetate and

lead citrate, stabilised by carbon evaporation and examined using an A.E.I. EM 801 electron microscope at an accelerating voltage of 60 kv (Cheng et al. 1980).

Experimental Design and Statistical Analysis

The overall design for the *in sacco* experiment was a completely randomized design analyzed as a split plot in time with tannin treatment (low or high) as the whole plot factor and sampling time as the subplot factor. Replications were nested within sampling time. The *in vitro* experiment was a completely randomized design which was analyzed over days using a split plot model with days as the whole plot factor and tannin treatment as the subplot factor. The replications were nested within days (Steel and Torrie 1980).

C. RESULTS

Results for *in vitro* digestion of the two strains of birdsfoot trefoil differing in tannin content are shown in Table II-1. The HT strain contained an average of five times more tannin, on a DM basis (plant to plant range: 0.3 to 17.8%), and showed a lower ($P<0.01$) DM disappearance (45.7 vs 54.4%) in 24 h than the LT strain (plant to plant range: 0.1 to 4% tannin). Because of variations in tannin content due to different plant batches used and day-to-day differences in rumen inocula (Fay et al. 1981), there was an interaction between the strain effect on DM digestion and days of experiment. The means for DM disappearance were different for twelve experiments out of thirteen ($P<0.01$ for eleven experiments, $P<0.08$ for one experiment) and the remaining experiment showed the same tendency but was not different.

A lower ($P<0.01$) gas pressure (50.9 kPa vs 58.0 kPa) developed during microbial digestion of the HT strain (Table II-1). There was no interaction between the effects of strain and days of experiment. A lower ($P<0.05$) VFA concentration (28.0 $mg.mL^{-1}$ vs 30.9 $mg.mL^{-1}$) was found in the rumen fluid after incubation with the HT strain of birdsfoot trefoil. There was no interaction between the effects of strain and days of experiment.

Table II-2 shows the results obtained when considering only the experimental days on which all three variables were determined, in order to relate DM disappearance to gas and VFA productions. The results illustrate that a reduction of 7.9% in VFA production and of 11.7% in gas pressure reflected the 19.5% less DM disappearance from the HT strain. Neither the increase in the microbial mass nor the production of non VFA (succinate, malonate, lactate), which are other possible fates of forage DM as a result of microbial digestion

Table II-1. Extent of *in vitro* dry matter disappearance, gas pressure increase and volatile fatty acid (VFA) production of birdsfoot trefoil strains after 24 h incubation

	High tannin		Low tannin		No. of observations/ treatment	Difference between means
	Mean	SEM+	Mean	SEM		
Tannin (% DM)	4.9	0.3	1.0	0.1	113	**
Dry matter disappearance (%)	45.7	0.4	54.4	0.4	84	**
Gas pressure increase (kPa)	50.9	11.0	58.0	11.0	42	**
VFA production ($mg.mL^{-1}$)	28.0	1.2	30.9	0.8	58	*

+Standard error of the mean.

**Significantly different, $P<0.01$.

*Significantly different, $P<0.05$.

Table II-2. Extent of *in vitro* dry matter disappearance, gas pressure increase and volatile fatty acid (VFA) production for observations restricted to experimental days where all three variables were measured on birdsfoot trefoil forage containing two different concentrations of tannins

	High tannin			Low tannin			% difference between means	Difference between means
	Mean	SEM+	N	Mean	SEM	N		
Tannins (% DM)	5.4	0.6	57	1.3	0.1	52		**
Dry matter disappearance %	41.7	0.6	34	51.8	0.6	34	19.5	**
Gas pressure increase (kPa)	48.4	1.3	34	54.8	1.3	34	11.7	**
VFA production ($mg.mL^{-1}$)	26.4	1.6	34	28.7	1.6	34	7.9	NS

+Standard error of the mean.

N = No. of observations per treatment.

**Significantly different, $P<0.01$.

NS = Not significantly different, $P > 0.05$.

(Hungate 1966), were determined in the present study. They could, however, explain part of the DM disappearance (%) that was not recovered in VFA or as gas production.

The results of the *in sacco* digestion studies show that DM loss from nylon bags was lower ($P<0.01$) for the HT strain (range 0.3 to 17.8% tannin) than for the LT strain (range 0.3 to 4.0%) after 8, 12 and 24 h of incubation (Table II-3). After 4 h, both strains were digested to almost the same extent. After 8 h, there was 12.5% difference (expressed as the % difference between two means also reported in %) between the two strains; after 12 h, the difference reached 17.3%, and then dropped to 9.7% after 24 h, at which time 74% of the plant material of the LT strain had been digested away (Fig. II-1). The tannin content was higher in the *in sacco* studies because two-year old birdsfoot trefoil plants only were used in these experiments, and, as reported earlier, these plants had higher tannin concentrations than the new seedlings. There was a significant interaction between the strain effect and days of experiment. The means for DM loss over all incubation times for both strains were significantly different for seven experiments out of ten ($P<0.01$ for six experiments, $P<0.05$ for one experiment). The remaining experiments showed the same tendency but the differences between strains were not significant. This interaction might be explained by variations in tannin content between two-year old batches of plants used for *in sacco* digestion studies. The location and displacement of the bottles with attached bags in the reticulo-rumen are also subject to day-to-day variations. Bags ending up being trapped with food ingesta in the upper part of the dorsal sac, following rumen

Table II-3. *In sacco* dry matter disappearance (%) of birdsfoot trefoil strains

Treatment	Incubation time (h)			
	4	8	12	24
High tannin (9.0%)	16.1	29.3a[+]	40.2c	67.0e
Low tannin (1.4%)	17.4	33.5b	48.6d	74.2f
SEM[++]	0.7	0.7	0.7	0.7
N[+++] = 40				

[+]Values followed by different letters are significantly different at $P<0.01$.

[++]Standard error of the mean.

[+++]N = Total no. of observations per treatment at each incubation time.

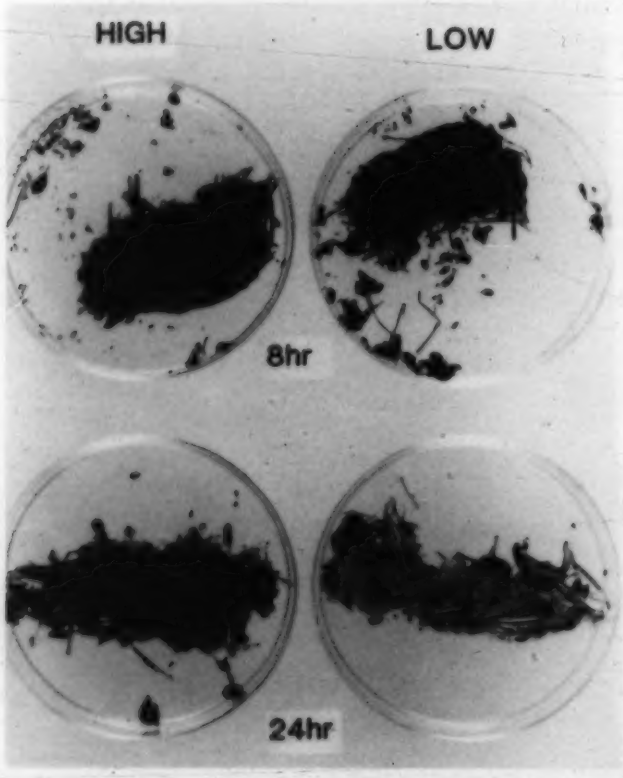


Figure II-1. *In sacco* digestion of high and low tannin strains of birdsfoot trefoil showing the almost complete disappearance of leaf material from the low tannin strain after 24 h.

contractions, for example, will be exposed to a larger microbial population than when present in the ventral part of the rumen which is more fluid and has a smaller microbial population (Forsberg and Lam 1977).

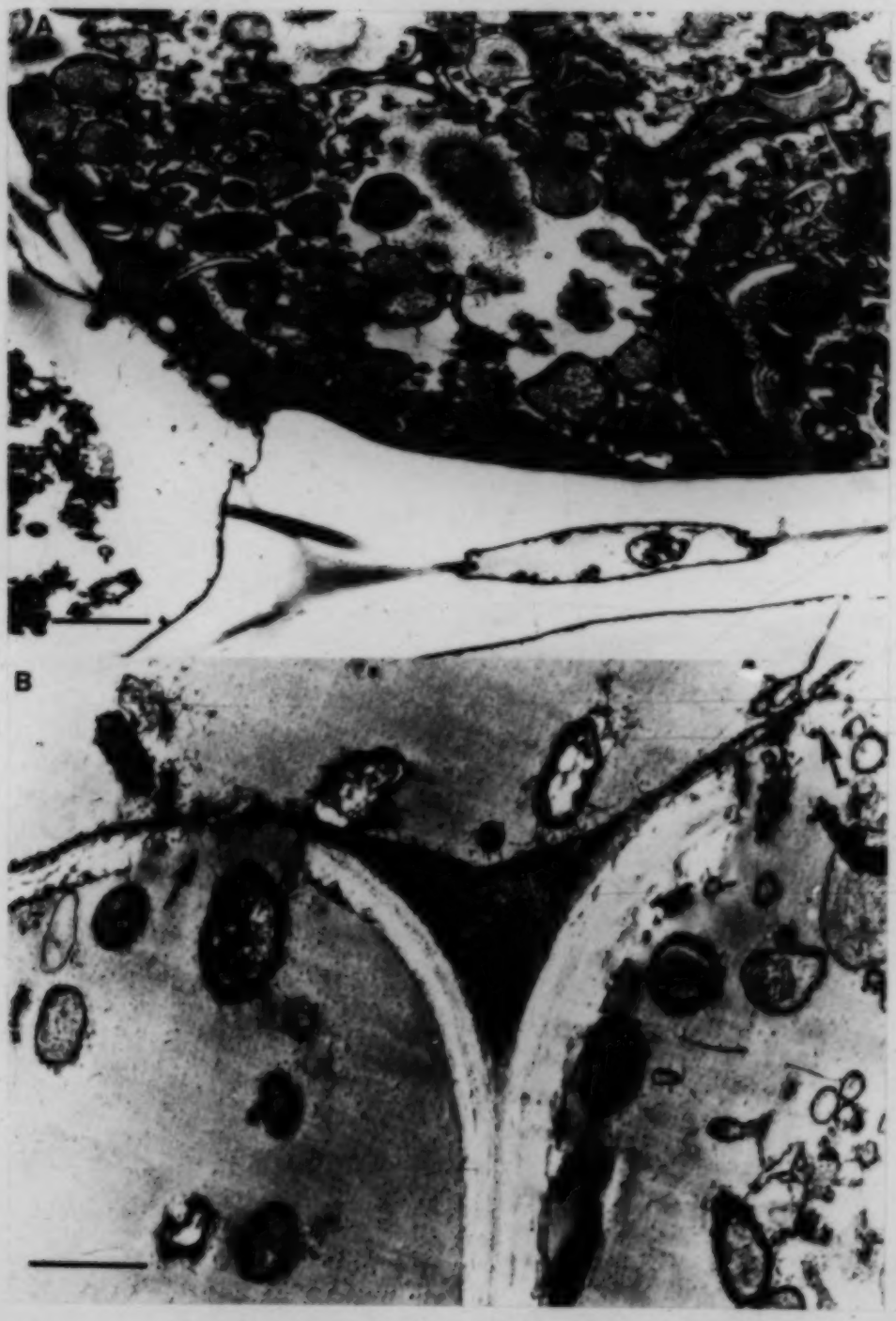
Direct examination of leaf and stem tissue of HT and LT strains of birdsfoot trefoil, by both SEM (Fig. II-2) and TEM (Fig. II-3), showed that rumen bacteria tended to form multiple adherent microcolonies on the HT material. Initially these bacterial microcolonies formed at the leaf and stem surfaces (Fig. II-2) and subsequently within intercellular spaces (Fig. II-3), but these populations did not seem to penetrate the plant tissues as effectively as did bacteria associated with the LT strains.

The SEM figures are representative of the majority of the eighteen specimens of HT or LT leaves or stems withdrawn from nylon bags at different incubation times. Each specimen was observed at five different magnifications. The TEM figures are representative of the majority of the thirty specimens of HT or LT leaves or stems withdrawn from nylon bags at different incubation times. Each specimen was observed at four different magnifications. These figures do not provide any quantitative information, but visual confirmation of the quantitative data.

Figure II-2. Scanning electron microscopy (SEM) of the surfaces of leaves of the high (A) and low (B) tannin strains of birdsfoot trefoil exposed to digestion for 4 h *in sacco* in the bovine rumen. The bacterial colonization of the high tannin strain tends towards the formation of bacterial microcolonies (arrows) whose cells would be protected from the antibacterial effects of tannins. The bacterial colonization of the low tannin strain shows a wide variety of bacterial morphotypes in close contact with the leaf surface. (Bars = 5 um).

A
B

Figure II-3. Transmission electron microscopy (TEM) of ruthenium red-stained preparations of stems of high (A) and low (B) strains of birdsfoot trefoil exposed to digestion for 24 h *in sacco* in the bovine rumen. Bacteria associated with the tissues of the high tannin strain tended to form glycocalyx-enclosed microcolonies while those associated with the low tannin strain proliferated, penetrated most cell compartments, and initiated cellulose degradation (arrows). Bars = 1.0 um.


A
B

D. DISCUSSION

The important variability in tannin content of HT and LT strain comes partly from the utilization of two age groups of plants. However, the variability within each age group is still important. The isosynthetic populations used were not, by definition, homozygous for all other characters than tannin content, as would have been the case for inbreeding "isogenic lines". This heterogeneity within each population could explain plant to plant variation in tannin content, and also the fact that, when working with small population numbers, a shift in the characters can be expected simply due to a sampling effect. Hayes and Immer (1942) reported that the amount of coumarin varies considerably in different parts of sweetclover (*Melilotus*) plants, and therefore care should be used in collecting the sample of leaves from the same relative portions of the plants. In the present study, leaves were stripped off several stems from each plant without measuring the distance from the tip or bottom of the stem. If the different distribution of coumarin within parts of sweetclover plants applies to birdsfoot trefoil tannins, the sampling method used in this study could have been the major reason for the discrepancy in tannin content among plants of the same strain.

The negative effect of tannin content on *in vitro* DM disappearance reported in this study agrees with results for crownvetch (Burns and Cope 1974), sericea lespedeza (Cope and Burns 1971) and sorghum forage (Arora et al. 1975). Cope and Burns (1971) reported that a LT strain of sericea (2.7% tannin in leaves and stems on a DM basis) was consistently about 25% higher in *in vitro* DM digestibility than a HT

strain (4.5% tannin in leaves and stems on a DM basis). This difference is comparable to that of 19.5% observed for the HT strain of birdsfoot trefoil (5.4% tannin in leaves on a DM basis). Lower gas production and VFA production are consistent with reduced DM fermentation. McGinty (1969) found that tannin inhibited gas production in the rumen, while Singh (1977, 1978) reported that tannin inhibited VFA production in the rumen, and Sadanandan and Arora (1979) found that ruminal VFA decreased with increase in the tannic acid concentration in the diet.

The nylon bag digestion studies showed that the DM loss from the HT strain was consistently lower ($P<0.01$) after 8, 12, and 24 h of incubation in the rumen than that of the LT strain. A similar study was conducted by Donnelly and Anthony (1969, 1970), who used the nylon bag technique on two cuttings of LT and HT sericea plants. In their study, DM disappearance was done after 24 h of incubation in the rumen and was found to be 63.4% and 58.0% from the HT (6.1% tannin in leaves and stems) plants in 1966 and 1968 respectively, compared with 71.8% and 65.0% from the LT (2.6% tannin in leaves and stems) plants. The difference in *in sacco* DM digestibility between the two tannin strains was then 11.7% and 10.8% in 1966 and 1968, respectively. These results are similar to those obtained in the present study where *in sacco* digestible DM was 66.7% for the HT strain (9.0% tannin in leaves) after 24 h of incubation in the rumen compared with 74.0% for the LT strain (1.4% tannin in leaves). The difference in digestible DM between treatments was, therefore, about 10% (percentage difference between two means also reported in percent). Since sericea lespedeza leaves were found to have a considerably higher percentage of tannin

than stems (Donnelly and Anthony 1973), we can expect that tannin content analyzed in leaves only, as in the present study, would have been much higher, since the presence of stems dilutes effective tannin concentration in the material.

The activities of many enzyme systems are inhibited by tannins (Pridham 1963) and can be increased by removing tannins with polyvinylpyrrolidone (Sanderson 1965; Loomis and Battaile 1966). The inactivation of microbial exoenzymes is caused by tannin complexing with the enzyme protein and with the substrate (Benoit and Starkey 1968). Smart et al. (1961) and Cope and Burns (1971) reported that a water-soluble substance in the leaves of sericea lespedeza inhibited the enzymatic hydrolysis of cellulose and that the reduction in cellulase activity was proportional to the concentration of the inhibitor present. Lyford et al. (1967) observed that this inhibitor was a part of the tannin fraction and was shown to be a polymer of delphinidin. Because of the inhibition of enzymes by tannins, it is to be expected that digestion, by the ruminant microflora, of the fiber and protein of a forage containing tannin would be depressed (McLeod 1974).

Further reduction in bacterial activity may result from the tendency of these organisms to grow in glycocalyx-enclosed micro-colonies in the presence of HT concentration. This "defensive" mode of growth limits the secretion of bacterial exoenzymes and limits the proliferation of bacteria and their penetration of plant tissues (Cheng et al. 1980). These bacterial responses to HT may also contribute to the observed reduction of DM disappearance of HT strains *in vitro* and *in sacco*. Tannin vesicles in birdsfoot trefoil are located just under the epidermal layer, frequently adjacent to the stomata. This

is an ideal location to inhibit the invasion of microorganisms (Howarth et al. 1984). From the present study conducted on two isosynthetic strains of birdsfoot trefoil, newly developed and tested for the first time, we conclude that the condensed tannins present in birdsfoot trefoil are responsible for the significant decrease in DM digestibility of the HT fresh forage. It seems, therefore, important to improve the utility of desirable forages (bloat-free legumes) by reducing tannin levels, so that the ruminant would benefit from the more digestible substrate (John and Lancashire 1981), without however eliminating them totally because of their beneficial effect on bloat prevention and their role in increasing the amino acid supply to the animal (Barry et al. 1986; Waghorn et al. 1987). The better understanding of microbe-tannin interactions in the rumen is one step towards improving forage quality through genetic modification.

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III. EFFECT OF TANNIN CONTENT IN TWO ISOSYNTHETIC STRAINS OF BIRDSFOOT TREFOIL ON FEED DIGESTIBILITY AND RUMEN FLUID COMPOSITION IN SHEEP

A. INTRODUCTION

Birdsfoot trefoil is a perennial forage legume extensively grown for pasture and hay in the North-Eastern and North-Central United States and the eastern provinces of Ontario and Quebec (Seany and Henson 1970; Grant and Marten 1985). In Western Canada, birdsfoot trefoil is a special purpose legume which is more tolerant of spring flooding and acid soils than are alfalfa or sweetclover, but has lower drought resistance and is less winterhardy than other legumes (Goplen, personal communication). Birdsfoot trefoil is a bloat-safe legume and may find greatest use where bloat is a problem and in those areas where alfalfa cannot be grown because of inadequate drainage or excessive acidity (Goplen, personal communication). Its bloat-safe characteristic has been attributed to the presence of condensed tannins and to the high resistance of its cells to rupture (Lees et al. 1981; Howarth et al. 1982).

Along with their role in bloat prevention, tannins have been shown to influence forage digestibility. *In vitro* and *in sacco* studies have demonstrated that high concentrations of condensed tannins depress DM digestibility of *L. cuneata* (Donnelly and Anthony 1969, 1970; Cope and Burns 1971), fababeans (Marquardt et al. 1978) and birdsfoot trefoil (Chiquette et al. unpublished results). Gas production and VFA production during *in vitro* incubation were also found to be depressed by high concentrations of tannins in birdsfoot

trefoil (Chiquette et al. unpublished results). High concentrations of tannins (10.6% on a DM basis) in *L. pedunculatus* reduced DM intake and ruminal protein degradation, and depressed rumen digestion of soluble carbohydrates and hemicellulose (Barry and Duncan 1984; Barry and Manley 1984). Tannins were also associated with a lower live-weight gain in sheep (Barry 1985). However, Barry et al. (1986) established that at the level of 1.4% and 4.5%, on a DM basis, the condensed tannins of *L. pedunculatus* reduced degradation of protein in the rumen while causing little or no reduction in rumen carbohydrate digestion.

Grasslands Maku (*L. pedunculatus*) has been the main forage used in the feeding trials dealing with the effect of tannins on forage digestibility. With the exception of the study by John and Lancashire (1981), who used two cultivars of birdsfoot trefoil differing in tannin content (0.25% vs 1.45%), and observed a reduction in the rate of feed protein degradation in the rumen and increased protein flow from the stomach but no effect on OM digestibility in HT compared with LT birdsfoot trefoil, LT and HT Lotus were produced by either spraying forage with polyethylene glycol (PEG), which selectively binds to tannins and prevents them from binding proteins, (Barry et al. 1986) or infusing it intraruminally (Waghorn et al. 1987). However, PEG treatment produces two side effects: a) an increase in the apparent concentration of lignin and b) an increase in the concentration of water-soluble carbohydrates in the plant and a reduction in its rate of degradation in the rumen (Barry et al. 1986). Condensed tannins and lignin are both polyphenolic compounds, and their monomers are synthesized in plants by the same shikimic acid biochemical pathway.

It is therefore probable that PEG binds to lignin by the same mechanism as it binds to condensed tannins, thus accounting for the increased concentration of 'artefact' lignin following PEG treatment. The effects of PEG treatment on water-soluble CHO are more difficult to explain. The most logical explanation according to Barry et al. (1986) is that PEG in some way inhibits enzymes involved in the degradation of this component, thus accounting for its increased concentration in the plant and reduced rumen degradation when the forage was sprayed with PEG.

The development of two isosynthetic strains of birdsfoot trefoil, differing only in their tannin content, following the method of Goplen (1969), has recently made it possible to study in feeding trials the effects of condensed tannins on the digestion of this forage. This study reports overall digestibility and digestibility at the rumen level of two isosynthetic strains of birdsfoot trefoil containing 1% and 3% (DM) condensed tannins respectively.

B. MATERIALS AND METHODS

Forage and Animals

LT and HT birdsfoot trefoil were grown in an irrigated field (0.25 ha) which had been divided in four equal longitudinal sections, alternating in both strains. The nature of these two isosynthetic strains has been outlined in detail in the Plant Material section of Chapter II. Prior to the start of the feeding trial, the field was randomly sampled to test both strains for tannin content differences and verify that the two strains effectively differ in tannin concentration. The qualitative vanillin spot test was used by crushing leaves between two layers of Whatman 0.16 mm chromatography paper, following the method of Sarkar and Howarth (1976).

Prior to, and following the feeding trial, *in sacco* digestibilities were determined on the upper halves of fresh forage plants that had been cut into 1-cm segments. Fifteen grams of cut forage were placed into 9 x 16 cm nylon mesh bags (Nitex No. 130, 10-N, 0.133 mm opening) which were suspended in the rumen of a fistulated steer fed cubed alfalfa hay. Triplicate bags of LT and HT birdsfoot trefoil were withdrawn after 4, 8, 12 and 24 h and washed with tap water. The digesta were dried (70°C overnight) and weighed for determination of DM disappearance.

Eight weeks before commencement of the study, thirteen Suffolk wethers were fitted with black, natural rubber cannulae having a 3-cm diameter opening, and were put on orchardgrass pasture during the recovery period.

Feeding Period

The feeding trial extended from June 1 to September 27 and was divided into three feeding periods. The first feeding period (June 1 to July 18) corresponded to the first growth of birdsfoot trefoil. The second feeding period (July 24 to August 29) corresponded to the first five weeks of the regrowth. The third feeding period (August 30 to September 27) corresponded to the last four weeks of the regrowth and was used for crossover of HT and LT feeding among the experimental animals. The first ten days of feeding periods one and three allowed adaptation of sheep to the respective strain they were assigned, while the adaptation period between the first and second feeding period was five days. At the end of the digestibility study, all sheep were offered cubed alfalfa hay (1.5% DM of their body weight) for two weeks following a 10-day adaptation period, in order to correct for intrinsic digestibility variation among sheep.

At the vegetative to mid-bud stages of growth, forage was cut long using either scythes or a Jari mower (Jari Division of Year-a-Round Cab Corporation, Mankato, Minnesota), and fed in long form to ten sheep (selected from the thirteen that were put on orchardgrass) housed indoors in individual pens or metabolic crates. Animals received birdsfoot trefoil at 1.3% of their body weight (DM basis) once daily. The feed remaining after 3 hours was removed and weighed. Rumen fluid samples (200-300 ml) were collected from eight sheep (two sheep were kept as spare ones), before feeding (0 h), 3 h and 6 h after feeding. Each sample was strained through two layers of cheesecloth to remove large particles. Daily samples of fresh forage fed to the animals as well as daily feces samples were oven-dried (45°C, 48 h for feces and

24 h for forage) and stored for further analysis. Part of the forage samples were freeze-dried for tannin determination.

Laboratory Analyses

Rumen fluid variables. Chlorophyll, pH and supernatant protein N determinations were performed between samplings while samples for NH_3-N determination, protozoal count, and VFA analysis were stored at 4°C and analyzed later. Rumen fluid pH was measured within 15 min of collection.

For chlorophyll content measurements, duplicate 0.5-mL subsamples of rumen fluid were combined with 2.0 mL of 80% acetone in a centrifuge tube. The mixture was left in the dark, at room temperature, for approximately 10 min, then centrifuged at 3020 x *g*. The supernatant was decanted and its absorbance read at 652 nm. The single wavelength method of Bruinsma (1963) was used to calculate chlorophyll concentration.

The Bio-Rad (Bio-Rad Chemical Division, 2200 Wright Avenue, Richmond, California 94804) Protein Assay was used for soluble protein quantification in the supernatant after centrifugation of whole rumen fluid (4080 x *g*, 10 min) to remove particulate matter. This dye-binding assay is based on the observation that the absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 shifts from 165 nm to 595 nm when binding to protein occurs (Bradford 1976). This assay was favored over other protein assays because of its simplicity, its rapidity, and its insensitivity to most of the interferences (such as phenolic compounds like tannins) which limit the application of the Lowry assay. NH_3-N was assayed by the phenol-hypochlorite method (Weatherburn 1967).

Sample preparation for protozoal count was carried out by diluting 1 mL of rumen fluid in 5 mL of methylgreen-formalin-saline (MFS) solution which was composed of 0.06% (wt/vol) methylgreen and 0.8% (wt/vol) NaCl in 10% (vol/vol) formalin, to fix and stain the nuclei of ciliates for preservation. The samples were sent for examination to the Department of Parasitology, Nippon Veterinary and Zootechnical College, Musashino, Tokyo 180.

For VFA determination, rumen samples (2 mL) were deproteinized overnight with 24% (wt/vol) m-phosphoric acid in 5N sulfuric acid (0.4 mL) and centrifuged at 7700 *g* for 30 min. The supernatant was thoroughly mixed with an equal volume of crotonic acid (30mM) as an internal standard. A 1-µL subsample was injected into a column of the type 6% free fatty acid phase (FFAP, Porapak 80/100) on a Varian model 3700 gas chromatograph, equipped with a Shimadzu chromatopac C-RIB data processor.

Tannin content and feed digestibility measurements. Freeze-dried birdsfoot trefoil leaf samples were analyzed for tannin concentration by the quantitative vanillin-HCl method based on Dalrymple's (1982) description. DM was determined by drying 100 grams of fresh forage for 24 h and daily feces for 48 h in a forced-air oven at 45°C. Oven-dried material was ground in a Wiley Mill through a 1-mm mesh screen and submitted to the Kjeldahl method (AOAC 1980) for CP determination, and to the methods of Goering and Van Soest (1970) for ADF and OM measurements.

The chemical composition (%) of the birdsfoot trefoil strains, collected throughout the regrowth, is shown in Table III-1. With the exception of total condensed tannins, for which the NT strain contained

approximately three times as much, and the CP which was slightly greater in the LT strain, the other components were found in similar proportion in both isosynthetics.

Statistical Analyses

Results of pH, chlorophyll and protein were averaged over periods of eight days in order to counterbalance daily variation, whereas for NH_3-N, protozoa count and VFA the sample numbers were not sufficient to allow regrouping, therefore, statistical analyses were performed over dates instead of periods. Rumen fluid variables were analyzed as a split plot in time with treatment (LT or HT) as the main plot factor and sampling time as the subplot factor. Sheep were nested within treatment and time. Tukey's test was used to compare more than two means (Steel and Torrie 1980).

The digestibilities of both forage strains were estimated over each 8-day period. The digestibility coefficients for each sheep, when fed alfalfa hay, were used as covariables to adjust for intrinsic digestibility variation. Digestibilities of forage components (DM, OM, CP and ADF) were determined for each sheep during the period they were all fed cubed alfalfa hay. Digestibility measurements for each component of trefoil were averaged over sheep. The average values of digestibility for each of the following trefoil components: DM, OM, CP and ADF, were used as reference values. For each individual sheep, the digestibility of each of the forage components was divided by its respective reference value, hence providing a relative digestion rate for each sheep and each forage component. Each sheep digestion rate was entered as a weight in a weighted analysis of variance (Steel and Torrie 1980).

Table III-1. Mean composition (% of DM) of the two birdsfoot trefoil strains (collected throughout the regrowth)

	Tannin content							Difference between means
	High	N*	Range	Low	N	Range	SEM**	
Condensed tannins	3.2	39	(1.6-6.5)	1.1	38	(0.7-1.6)	0.2	**
Dry matter	20.9	90		20.8	90		0.3	
Organic matter	90.0	30		90.0	30		0.4	
Crude protein	15.1	30		16.2	30		0.4	
Acid detergent fiber	31.1	30		32.1	30		1.0	

*No. of observations.

**Standard error of means.

**Significantly different, $P \leq 0.01$.

C. RESULTS

The immature LT or HT plant material (beginning of the first growth and regrowth) was digested to the same extent when incubated in the rumen of a steer for 24 h, using *in sacco* digestion technique (Table III-2). When sampled on the last day of first feeding period, the DM content had increased substantially, and its digestion was reduced from 80% to 50%.

The HT diet did not seem to affect forage acceptance by the animals during the adaptation period and throughout the experiment. Sheep receiving HT birdsfoot trefoil had an average daily intake of 25.37 g of DM/kg of metabolic weight ($WT^{0.75}$), compared with 25.55 g of DM/kg of $WT^{0.75}$ for sheep fed LT birdsfoot trefoil.

Table III-3 summarizes the results from rumen fluid analysis at each sampling period. The sheep fed HT birdsfoot trefoil showed a chlorophyll concentration slightly but not significantly lower at each sampling time. The highest chlorophyll concentration was recorded in the rumen fluid samples prior to feeding. It declined by 40% of the 0 h value, 3 h after feeding and climbed by 25-30% of the 3 h value, 6 h after feeding. The sheep receiving the HT strain showed a lower ($P<0.01$) supernatant protein N concentration at each sampling time. For both HT- and LT-fed animals, the pattern with time was similar to that recorded with chlorophyll, although the variation of protein with time was somewhat smaller (35% decrease and 15% increase between 0 h and 3 h, and 3 h and 6 h, respectively) than for chlorophyll. As for chlorophyll, the protein concentration was different ($P<0.05$) at each sampling time for both birdsfoot trefoil strains. NH_3-N results

Table III-2. Digestion of birdsfoot trefoil strains (stems and leaves) upon 24 h incubation in nylon bags suspended in the rumen of a steer

Experiments	Tannin content	Dry matter content (%)	Apparent dry matter digestion (%)	N+	SEM++
1	High	16.4	80.9	3	0.6
	Low	16.1	79.7		
2	High	27.5	48.2	3	3.0
	Low	27.9	54.3		
3	High	16.9	79.5	3	0.2
	Low	16.7	79.2		

+No. of bags per treatment at each experiment.

++Standard error of means.

Experiment 1: first day of first feeding period; Experiment 2: last day of first feeding period; Experiment 3: first day of second feeding period.

Table III-3. Rumen fluid variables measured before feeding, 3 and 6 h after feeding, in sheep fed high and low tannin birdsfoot trefoil (average over the three feeding periods).

Variables	Sampling time (h)	Tannin content: High	N+	Low	N	SEM++	Difference between treatments
Chlorophyll ($\mu g\ mL^{-1}$)	0	80.7_a	192	81.0_a	224	1.4	NS
	3	47.4_b	192	48.5_b	224	1.4	NS
	6	59.7_c	192	64.3_c	224	1.4	NS
Supernatant protein N ($\mu g.mL^{-1}$)	0	269.0_a	192	297.0_a	224	4.2	**
	3	173.0_b	192	197.0_b	224	4.2	**
	6	198.0_c	192	223.0_c	224	4.2	**
NH_3 ($\mu g.mL^{-1}$)	0	166.0	66	162.0_a	75	7.2	NS
	3	167.0	72	211.0_b	79	6.4	**
	6	176.0	70	207.0_b	79	6.5	**
VFA ($mg.mL^{-1}$)	0	20.2_a	33	19.3_a	39	1.2	NS
	3	39.1_b	33	39.1_b	39	1.2	NS
	6	40.3_b	33	42.0_b	39	1.2	NS
pH	0	7.4_a	192	7.4_a	224	0.02	NS
	3	6.7_b	192	6.7_b	224	0.02	NS
	6	6.5_c	192	6.5_c	224	0.02	NS
Protozoa ($x10^4$).mL^{-1}	0	11.7_a	64	12.0_a	72	0.5	NS
	3	8.7_b	64	7.1_b	72	0.5	*
	6	7.9_b	64	7.2_b	72	0.5	NS

For each variable, values in same column having different subscripts are different, $P<0.05$.

+No. of observations.

++Standard error of means.

NS Not significantly different, $P>0.05$.

**Significantly different, $P<0.01$.

*Significantly different, $P<0.05$.

showed no difference between treatments prior to feeding, while at 3 h and 6 h after feeding, a lower ($P<0.01$) concentration was found in the rumen of the HT-fed animals. The sheep receiving the HT forage did not show significant variation in their ruminal NH_3-N level with sampling times, whereas an increase ($P<0.05$) in concentration occurred in the rumen of the LT animals 3 h after feeding and was unchanged 6 h after feeding. Ruminal VFA concentration and pH were similar in sheep given either LT or HT birdsfoot trefoil, although VFA concentrations consistently tended to be lower in the rumen of the HT-fed animals at 6 h after feeding. The level of VFA in the rumen of each treated group had doubled ($P<0.01$) 3 h after feeding and did not ($P>0.05$) change further at 6 h after feeding. When considered individually (Table III-4), each VFA, except isobutyrate and isovalerate, followed the same pattern with time as for the total VFA with a trend towards a lower concentration in the HT-fed animals. The pH values decreased ($P<0.01$) with time. Although protozoal count per mL of rumen fluid tended to be higher with the higher level of dietary condensed tannins in the 3-h and 6-h samples, the trend was significant only at 3 h. In both groups, the highest protozoal count was recorded prior to feeding. At the second sampling period, it was 40% and 25% of its initial value, in the LT- and HT-fed animals, respectively, and the 6 h protozoal count did not differ from the previous one recorded in each treatment. Within each rumen fluid variable, the specific effect of sampling time was found to be similar over days or periods of analysis.

The extent of apparent digestibility for each forage strain is presented in Table III-5. DM, OM, and ADF apparent digestibilities were not affected by tannin level. The averaged CP digestibility was

Table III-4. Individual volatile fatty acid (VFA) concentrations over sampling times

VFA ($mg.mL^{-1}$)	Sampling time (h)	Tannin content				
		High	N+	Low	N	SEM++
Acetate	0	2.3_a	33	2.3_a	39	0.1
	3	4.3_b	33	4.5_b	39	0.1
	6	4.4_b	33	4.9_b	39	0.1
Propionate	0	0.4_a	33	0.5_a	39	0.04
	3	1.1_b	33	1.2_b	39	0.04
	6	1.1_b	33	1.3_b	39	0.04
Butyrate	0	0.22_a	33	0.22_a	39	0.02
	3	0.38_b	33	0.40_b	39	0.02
	6	0.44_c	33	0.49_c	39	0.02
Isobutyrate	0	0.07	33	0.07	39	0.02
	3	0.04	33	0.09	39	0.02
	6	0.04	33	0.04	39	0.02
Isovalerate	0	0.07	33	0.05	39	0.01
	3	0.04	33	0.05	39	0.01
	6	0.04	33	0.04	39	0.01

a-c Means within individual volatile fatty acids with different letters are significantly different, $P<0.05$.

+No. of observations.

++Standard error of means.

Table III-5. Apparent digestibilities (%) of birdsfoot trefoil strains in sheep.

Components	Periods[+]	Tannin content: High	N[++]	Low	N	SEM[++]	Difference between means
Dry matter	1	67.6	20	66.8	20	1.9	NS
	2	63.3	32	66.3	32	2.0	NS
	3	55.3	24	57.9	38	1.9	NS
	4	67.8	27	65.9	45	1.9	NS
	$\bar{x}$	64.0	103	63.6	135	1.5	NS
Organic matter	1	70.9	20	70.0	20	2.1	NS
	2	65.6	32	68.6	32	2.2	NS
	3	62.4	24	66.1	38	2.1	NS
	4	74.4	26	73.8	45	2.1	NS
	$\bar{x}$	68.3	102	69.6	135	1.1	NS
Crude protein	1	67.5	20	78.0	20	3.2	*
	2	66.0	32	77.1	32	3.4	*
	3	71.4	21	73.6	33	3.2	NS
	4	77.2	20	78.4	30	3.2	NS
	$\bar{x}$	70.5	93	76.8	115	1.7	**
Acid detergent fiber	1	46.6	20	45.4	20	4.4	NS
	2	30.9	32	40.7	32	4.7	NS
	3	29.5	21	30.3	33	4.4	NS
	4	37.4	20	37.0	30	4.4	NS
	$\bar{x}$	36.1	93	38.3	115	2.3	NS

Each of periods 1 and 2 represent digestibility data averaged over 5 and 8 consecutive days, respectively, before crossing over.
Each of periods 3 and 4 represent digestibility data averaged over 8 and 9 consecutive days, respectively, following crossing over.
[+]No. of observations.
[++]Standard error of means.
NS = not significantly different, $P>0.05$.
*Significantly different, $P<0.05$.
**Significantly different, $P<0.01$.

approximately 8% less in the HT strain than in the LT strain. There was an interaction between the effect of tannin level on the digestibility of CP with periods, while the effect of tannin level on DM, OM and ADF digestibilities was the same over all periods of analysis.

When analysis of variance was repeated without employing digestibility coefficients, the means for each strain as well as their standard error showed very little change, which did not affect the statistical significance. This suggests that the individual digestibilities of sheep fed the same diet (alfalfa hay) were uniform and did not interfere with the sheep response to tannin level in the diet.

D. DISCUSSION

Lower tannin levels are reported in this population of birdsfoot trefoil (Table III-1) compared with the greenhouse population used in the study described in chapter II. According to Goplen (personal communication), genetic equilibrium was obtained in one generation of panmixy (random mating) and unless there was a selective influence, there should not have been any "genetic homeostasis" (redistribution of genes causing a gradual decline in the character for which selection was made). The change in environmental conditions would, most likely, have been the major factor for altered tannin concentrations when birdsfoot trefoil was grown in the field. Ross and Jones (1983) reported reduced tannin content in *L. pedunculatus* Cav. when grown outdoors compared to greenhouse conditions.

Preliminary experiments on the DM digestibility of each birdsfoot trefoil strain indicated that the *in sacco* digestibility of DM was not affected by tannin content when comparing strains at the same maturity. This finding is consistent with the absence of tannin effect on DM and OM apparent digestibilities as observed when both strains were fed to sheep. However, there was a noticeable reduction in DM digestibility as the forage matured. Quantitative tannin analyses performed on plant material collected throughout the growing season, revealed that the concentration of flavolans was a function of forage maturity, as reported by Burns (1966). Tannin concentrations in leaves (% of DM) of the LT and HT strains were 0.5 and 1.0, respectively, at the beginning of the first feeding period, compared with 0.9 and 3.4, respectively, towards the end of the same period.

Care must be exercised to differentiate between the effects of tannin activity and of lignification on the digestibility of mature forage compared with that of younger forage. Although tannins partly explain the low digestibility of mature forage, since the amount varies both during the season and during development, increasing in leaves of both annual and perennial plants as the season progresses (Swain 1979), they may not be the principal contributing factor. Hence, legumes undergo substantial stem thickening, and marked increases in stem weight take place before flowering (Walton 1983). Forage stems contain a large proportion of lignified tissues which resist microbial degradation (Akin 1984). As maturation takes place, lignin is deposited on the microfibrils and other cellulose structures in the secondary cell walls of plants. It will vary from about 2% of the DM in the young plant to 17% or more in a fully mature plant (Walton 1983). Therefore, the increase in tannin concentration with plant maturity might have played a role in the reduction of DM digestibility observed in the present study, however, stem lignification was, most likely, the principal contributing factor.

Rumen Fluid Variables

The similar chlorophyll concentrations in the rumens of sheep fed either strain (Table III-3) indicate that plant cells released their chloroplast particles to almost the same extent following disruption as a result of mastication and microbial activity (Cheng et al. 1980). Plant protein solubility and microbial deamination appeared to be significantly altered as a result of the different tannin contents of the two strains. Soluble protein content of rumen fluid and possibly

microbial deamination, as partially reflected by the lower NH_3-N concentration, were about 11% less in animals given the HT than in those given the LT birdsfoot trefoil. Previous studies (Driedger and Hatfield 1972; John and Lancashire 1981; Waghorn et al. 1987) reported that tannin-protein binding reduces protein solubilization in rumen fluid and, because of reduced substrate concentration, decreases ruminal deamination or proteolysis. John and Lancashire (1981) reported 27% less NH_3-N in the rumen fluid of sheep fed a HT (1.45% of DM) cultivar of *L. corniculatus*, neither time of sampling after feeding nor parts of plants that were analyzed for tannin content were mentioned, compared with 20% found in this study, in the 3-h samples (Table III-3). Barry et al. (1986) established that at levels of 1.4% and 4.5% tannins in leaves, on a DM basis, the condensed tannins of *L. pedunculatus* reduced degradation of protein in the rumen while causing little or no reduction in rumen carbohydrate digestion, which suggests the nearly total absence of microbial enzyme inhibition at this level of tannin.

Kurihara et al. (1968) observed an inverse relationship between the protozoal and bacterial population in the rumen, mainly due to competition for substrate. This suggests that there may have been fewer bacteria present in the rumen of the HT-fed animals possibly as a response to a lower availability of substrates, among which soluble proteins.

The highest concentrations of chlorophyll and supernatant protein N were detected in the rumen liquor prior to feeding (Table III-3). Similar results were reported by Kudo et al. (1985) when sheep were on a restricted diet of alfalfa selected for slow and fast initial rates

of digestion. Rumen turnover is known to be slow when intake is reduced (Owens and Goetsch 1984). The higher concentrations of chlorophyll and supernatant protein N found in the rumen fluid prior to feeding, as opposed to 3 h and 6 h after feeding, could be attributable to very low rumen fill encountered in the morning, which complicated sampling. Three hours after ingesting approximately 3 kg of forage containing an average of 80% water, the fluid level increased substantially so that the lower concentration appearing in the 3 h sample could, in fact, have been an effect of dilution. Bloat-safe forages have been found to have a higher resistance to plant cell rupture (Lees et al. 1981). This could have caused intercellular fluid to be released in the rumen faster than intracellular plant constituents. Consequently, the rate at which intracellular plant constituents were liberated in the rumen could have been slower than the rate at which fluid was leaving the rumen, this could be part of the explanation for the relative increase in chlorophyll and supernatant protein N concentrations found in the 6-h samples. However, marker studies would be essentials to provide more information for base of discussion. Clarke (1965) also found a decrease in protozoal count after feeding cows once daily on a diet of fresh red clover which he attributed to the changes in the volume of rumen contents during feeding. Kistner et al. (1962) reported that during feeding the count of bacteria per mL diminishes because of dilution by feed and saliva, but the fermentation rate is high because of the increased substrate. The count per mL ultimately increases after feeding (Hungate 1966). Quantitative estimates of salivary production indicate that 6 L of saliva enter the reticulo-rumen in

sheep daily. There are also indications that water may be secreted through the walls of the rumen, but quantitative estimates of this fraction are uncertain (Church 1969). Majak et al. (1983), in an attempt to explain similar findings in bloating cattle fed alfalfa herbage, mentioned the possibility that chlorophyll concentration was affected by release of frothy rumen contents when cannulae were opened for the 2-h sample collection, which would imply that the chloroplast particles were floating in the rumen.

No explanation can be offered for the pattern of NH_3-N concentration with time because many processes are responsible for NH_3-N fluctuations in the rumen, such as: production of NH_3-N by transfer of urea from the blood to rumen fluid (Gartner 1963), salivary urea entering the rumen (McDonald 1948), deamination of peptides, amino acids, amides, purines and pyrimidines (Leng and Nolan 1984), nitrite reduction (Leng and Nolan 1984), from which NH_3-N mainly derives in the rumen, and loss due to: absorption through the rumen wall (McDonald 1948), passage from the rumen to the abomasum (McDonald 1948) and incorporation in the synthesis of bacterial and protozoal protein (Boggs 1959).

At the sampling times they were measured, the VFA concentrations as well as the pH did not differ significantly with tannin content. As Hungate (1966) reports, although the concentration of the VFA's in the rumen is a function of production, it is not directly proportional and can thus serve only as a very rough index to the rate of production. The decrease in pH following eating reflected the changes in the quantities of organic acids produced in the digesta. VFA are readily absorbed through the rumen epithelium and also leave the rumen

by passage to the omasum (Hungate 1966), so that 24-h concentrations were low. We would have expected a lower pH in the rumen of the LT-fed animals than in HT-fed animals as a consequence of VFA formation during the process of bacterial deamination of soluble proteins since they are, with carbohydrates, VFA substrates. Most reports on the subject indicate that pH in the rumen will vary with the location of samples (Bryant 1964; Lane et al. 1968). Another possibility is that the amount of VFA produced from protein degradation was not sufficient to influence pH.

Digestibility

Barry and Manley (1984) reported depressed OM digestibility when feeding sheep *L. pedunculatus* containing 10.6% tannins. John and Lancashire (1981), however, working with two cultivars of *L. corniculatus*, showed that condensed tannins present in concentrations of 0.25% and 1.45% (parts of plants on which measurements were made was not specified), on a DM basis, had no effect on OM digestibility (71.3% vs. 70.5%). Similarly, Barry et al. (1986), when feeding sheep *L. pedunculatus* containing 4.5% and 1.4% tannins (in leaves), did not measure an effect on OM digestibility, nor did we for our two strains of birdsfoot trefoil containing comparable tannin concentrations (3.2% of DM vs 1.1% of DM in leaves). Barry and Forss (1983) proposed the concept of free-tannins which they defined as representing the total condensed tannins that have exceeded the binding capacity of plant proteins and are water soluble. Free-tannins could be a detrimental factor by possibly reacting with and precipitating proteins of the gut wall and extracellular carbohydrate-

degrading enzymes secreted by rumen bacteria (Barry and Forss 1983). These authors predicted that the free-tannin fraction would be zero when total tannins represent 1.8% of the DM of *L. pedunculatus*; at this concentration, the majority of tannins are bound to the plant proteins. This may explain why the main effects of tannins, in the present study, were the altered supernatant protein N and NH_3-N concentrations.

With respect to apparent CP digestibility, Waghorn et al. (1987) feeding sheep with *L. corniculatus* containing 2.2% tannins (parts of plants on which measurements were made was not specified) vs the same forage after treatment with PEG, found total apparent digestion of protein to be 70% and 78% respectively as compared to 70% and 77% found in this study and 71% and 56% reported by Barry and Manley (1984) when feeding *L. pedunculatus* containing 4.6% and 10.6% condensed tannins (parts of plants on which measurements were made was not mentioned). As reported earlier in the present study, there was an interaction between the effect of tannin level on the digestibility of the CP and the periods (eight days each) over which digestibility data were averaged. This interaction might have been caused by the fact that periods one and two of digestibility analysis started fifty-six days after sheep were adapted to their respective birdsfoot trefoil strain while periods three and four of digestibility analysis immediately followed a ten-day adaptation after birdsfoot trefoil strains were switched among experimental sheep (crossing over). The shorter time during which the sheep were fed their respective birdsfoot trefoil strain after the crossing over took place might explain the equilibrium that seemed to have established in CP digestibility

between sheep fed LT and HT birdsfoot trefoil, following crossing over (Table III-5). The small difference on a % basis in the apparent digestibility of CP between the two strains might reflect the greater post-ruminal flux and absorption of N that was reported by Barry and Manley (1984) and Waghorn et al. (1987), from the HT Lotus due to the reversibility of the tannin-protein complex which dissociates and releases proteins at pH < 3.0 and at pH > 8.0 as is encountered in the abomasum and small intestine, respectively (Jones and Mangan 1977). It could also be attributable to an increase in microbial-N in the feces of the LT-fed animals as was found by John and Lancashire (1981) who reported 15% more microbial-N leaving the stomach of sheep fed LT birdsfoot trefoil.

Bloat Occurrence

There was no indication of bloat in this study. This observation is preliminary in that the amount of forage intake and the number of sheep fed were inadequate for conclusive results. Furthermore, the similarities in the patterns of ruminal chlorophyll and supernatant protein N with time reported in the present study and in that of Majak et al. (1983), where measurements were performed on bloated cattle, raises the possibility of bloat potential at this low level of tannins. Thus, there is a need for grazing trials to be carried out in order to assess both strains with respect to their bloat potential.

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IV. GENERAL CONCLUSIONS

When present at an average concentration of 1% or 3% of birdsfoot trefoil DM, condensed tannins did not cause any detrimental effect on the forage DM digestibility *in vivo*. On the contrary, the highest tannin content appeared to depress soluble protein degradation at the rumen level. The ideal dietary concentration would seem to be the minimum amount of condensed tannin necessary to render the plant protein insoluble. Higher concentrations are likely to prove detrimental, especially if the tannin-protein complex formed from disintegration of such plants releases high proportions of free-tannins. Barry (1985) suggested an ideal condensed tannin concentration of 3-4% DM for *Lotus pedunculatus*, while Donnelly and Anthony's data (1970) suggest a tannin threshold, in relation to digestible DM, between the LT and HT groups of sericea lespedeza, near the upper limit of their LT group (i.e., 3.4% tannin on a DM basis). Results obtained from the present study would also seem to agree with these threshold levels in the case of birdsfoot trefoil (same tannin molecular weight as *Lotus pedunculatus*; Barry and Manley 1986), although natural tannin content of birdsfoot trefoil being 0.2-3.0% on a DM basis (Barry and Reid 1984) rarely attains detrimental levels, unless environmental conditions favor their production (e.g., low fertility soils under cold conditions).

In view of this, it seems that breeders effort should not be looking at eliminating tannin completely but at improving the utilization of bloat-free legumes by reducing tannin levels to a threshold value (approximately 3-4% of DM) below which DM

digestibility is no longer reduced significantly while the bloat-safe characteristic of the forage is still maintained (Jones and Mangan 1977; Barry et al. 1986). Such material would have the additional merit of higher levels of bypass protein and better utilization of the protein-tannin complex in the small intestine (John and Lancashire 1981; Barry et al. 1986; Waghorn et al. 1987). If tannins should be transferred from bloat-safe to bloat-causing forages, it would also be necessary to restrict their concentration near the threshold value. Although changes in constituents obtained through selection procedures may be small, it must be remembered that any incremental increase in energy intake above maintenance (due to either enhanced DM intake, digestibility, or rate of passage) constitutes energy that remains nearly all available for productive purposes. Efforts to obtain such improvement in the nutritive value of forages must be strengthened and encouraged (Burns 1978).

This study, resulting from the cooperation between plant breeders and researchers in feed utilization by ruminants, provides an example of the control of microbial digestion rates by the genetic manipulation of plants, and suggests an era in which forages will be bred to optimize their utilization by rumen microbes and thus their provision of nutrients to ruminants.

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APPENDIX

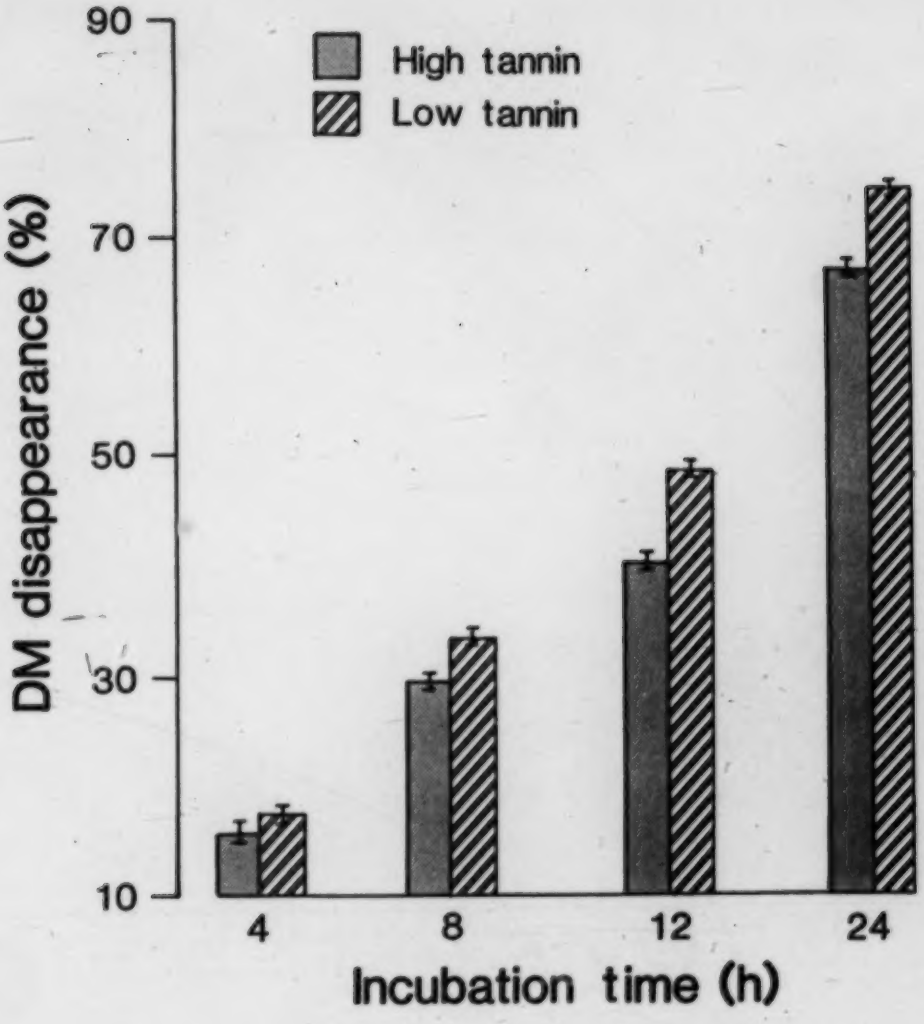

Figure A-1. *In sacco* dry matter (DM) disappearance (%) of birdsfoot trefoil strains.

Table A-1. Daily estimates of tannin content in the birdsfoot trefoil strains fed to the sheep.

Julian day	Field sites*		Parts of Plants analyzed	Tannin content (g/100 g DM) Birdsfoot trefoil	
				Low tannin	High tannin
1st feeding period					
163	LO1-1	Hi2-2	Leaves + Stems	0.78	1.35
166	LO1-1	Hi2-2	"	0.39	0.89
168	LO1-1	Hi2-2	"	0.46	1.64
169	LO1-1	Hi2-2	"	0.46	0.57
171	LO1-1	Hi2-2	"	0.43	0.49
174	LO1-1	Hi1-1	"	0.28	0.43
175	LO1-1	Hi1-1	"	0.00	0.52
176	•	Hi1-1	"	•	0.38
177	LO2-2	Hi2-2	"	0.51	1.20
178	LO1-2	Hi1-2	"	0.73	0.82
181	LO1-2	Hi1-2	"	0.22	0.63
182	LO2-2	Hi2-2	"	0.26	0.58
183	LO1-2	Hi1-2	"	0.29	2.82 (Leaves only)
184	LO2-2	Hi2-2	Leaves	0.93	1.70
185	LO1-2	Hi1-2	"	0.76	2.51
188	LO2-2	Hi2-2	"	1.02	3.10
189	LO1-2	Hi1-2	"	0.67	6.02
192	LO2-2	Hi2-2	"	0.88	3.27
195	LO1-2	Hi1-2	"	0.89	2.98
196	LO2-2	Hi2-2	"	1.11	2.45
198	LO2-2	Hi2-2	"	0.70	2.39
2nd feeding period					
205	LO2-1 / LO1-1	Hi2-1	"	0.67	1.92
209	LO2-1	Hi2-1	"	1.05	2.19
210	LO2-1	Hi2-1	"	1.04	2.85
213	LO1-1	Hi2-1	"	1.58	4.02
217	LO1-1	Hi2-1	"	0.70	3.86
218	LO1-3	Hi1-3	"	1.29	4.00
219	LO1-3	Hi1-3	"	1.64	3.38
220	LO1-3	Hi1-3	"	1.42	4.66
223	LO1-3	Hi1-3	"	0.98	4.56
224	LO1-3	Hi1-3	"	1.11	2.88
225	LO1-3	Hi1-3	"	1.30	4.53
227	LO1-3	Hi1-3	"	1.40	2.57
230	LO2-3	Hi2-3	"	0.98	2.37
231	LO2-3	Hi2-3	"	1.04	5.40
232	LO2-3	Hi2-3	"	1.93	6.48
237	LO2-3	Hi2-3	"	1.20	5.47
238	LO2-3	Hi2-3	"	3.03	2.34
239	LO2-3	Hi2-3	"	1.42	3.92
240	LO2-3	Hi2-3	"	0.85	3.03

Table A-1 Continued

Crossing-over (or 3rd feeding period)

252	LO1-3	Hi1-3	0.89	3.36
253	LO1-3	Hi2-3	0.86	1.63
255	LO1-2	Hi1-2	0.73	2.31
258	LO1-2	Hi1-2	0.15	2.13
259	LO1-2	Hi1-3	1.17	2.77
260	LO1-2	Hi1-2	0.82	2.19
262	LO2-2	Hi2-2	1.04	1.71
265	LO2-2	Hi2-2	0.79	2.56
266	LO2-2	Hi2-2	1.37	2.47
267	LO2-2	Hi2-2	1.13	3.04
268	LO2-2	Hi2-2	0.70	2.41

*Field sites:

North

	LO2-1	LO2-2	LO2-3
	Hi2-1	Hi2-2	Hi2-3
West	LO1-1	LO1-2	LO1-3
	Hi1-1	Hi1-2	Hi1-3

East

South

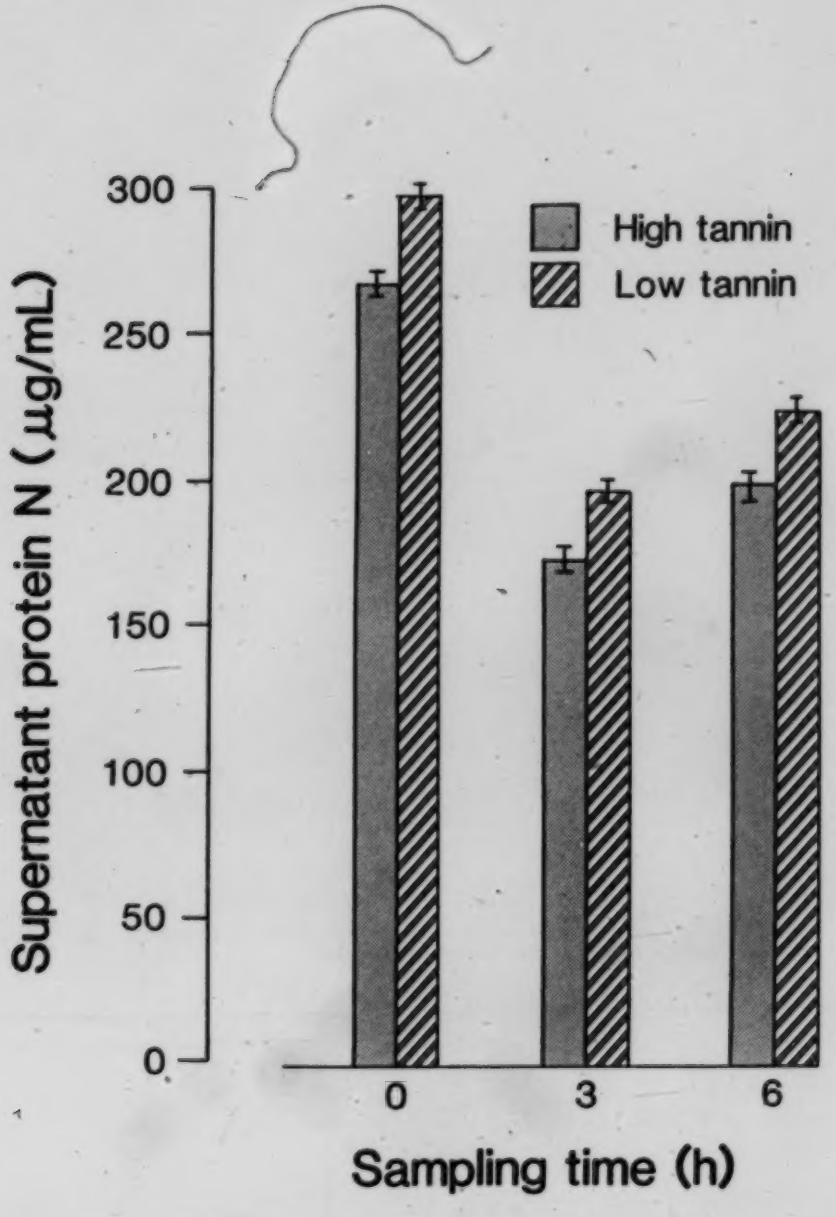

Figure A-2. Ruminal supernatant protein N concentration at 0, 3 and 6 h after feeding sheep with low and high tannin birdsfoot trefoil.

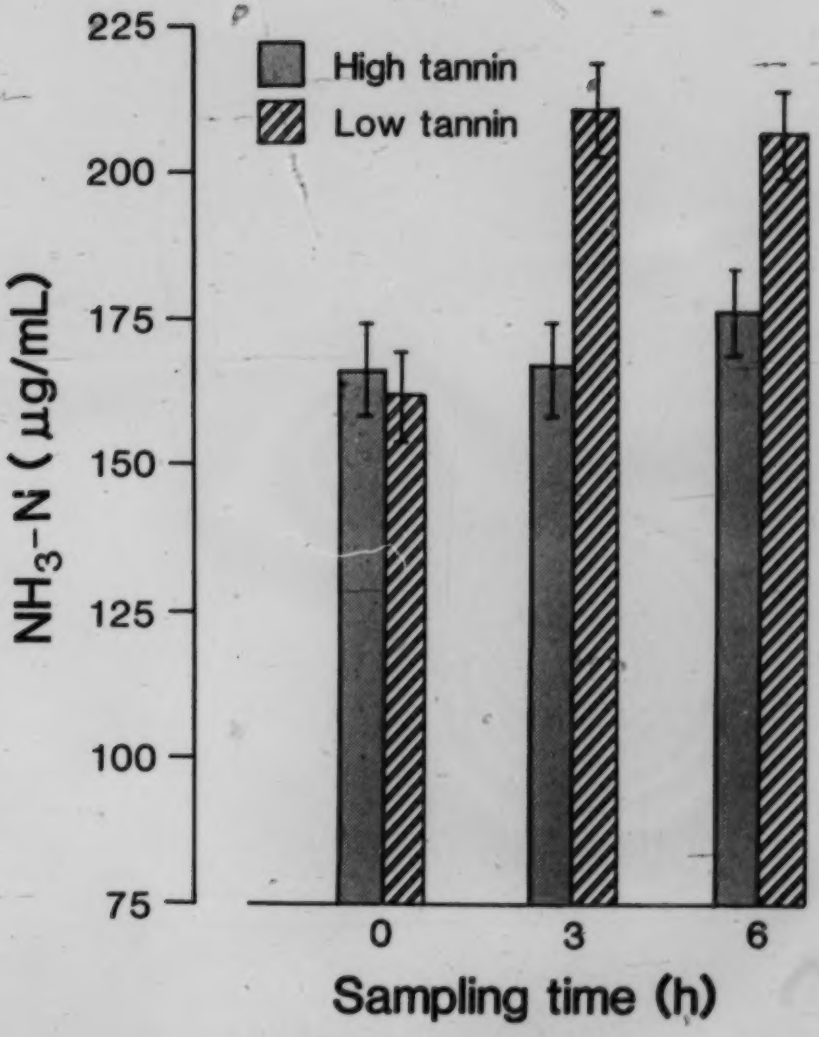

Figure A-3. Ruminal NH_3-N concentration at 0, 3 and 6 h after feeding sheep with low and high tannin birdsfoot trefoil.

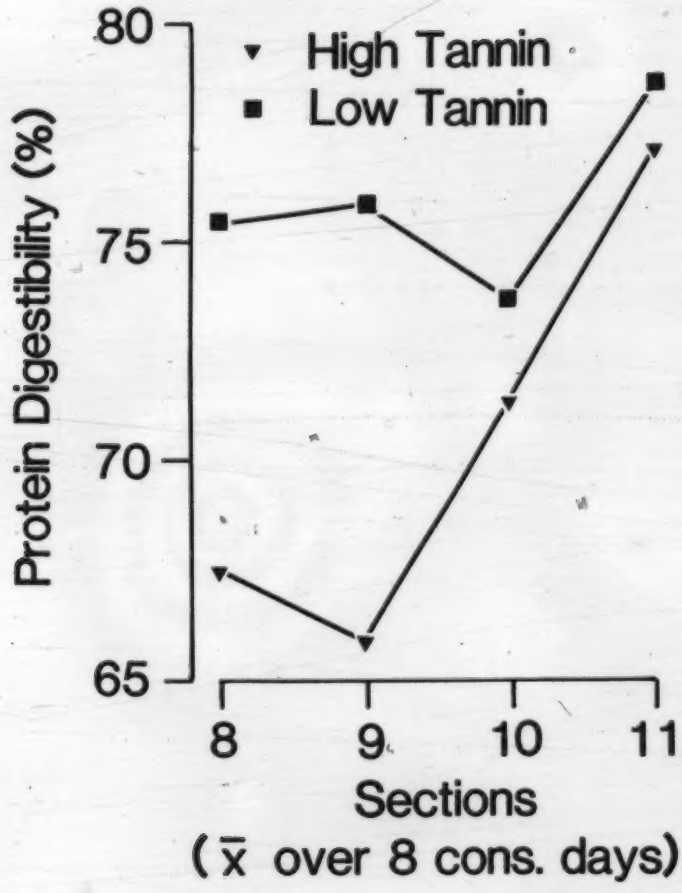

Figure A-4. Crude protein apparent digestibility with time. Section 10 corresponds to the first days following crossing over where treatments (low or high tannin birdsfoot trefoil) were switched between sheep and the period of adaptation on the new strain was shorter than in sections 8 and 9.